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(54) Title: ACTIVATED ENZYME-LINKED DETECTION SYSTEMS FOR DETECTING AND QUANTIFYING NUCLEID ACIDS, ANTIGENS, ANTIBODIES AND OTHER ANALYTES

(57) Abstract: Analytes bound to immobilized capture molecules by molecular biological or immunochemical reactions are detected or quantified with affinity liposomes containing encapsulated enzyme activators and surface-attached affinity components capable of specific binding to captured analytes or derivatives thereof in a structure restricted manner. Enzyme activators encapsulated in analyte-bound affinity liposomes are released by temperature- or detergent-mediated mechanisms and utilized for activation of solid phase-immobilized inactive enzyme molecules which in turn are utilized to generate colorimetric, fluorescent, chemiluminescent, or bioluminescent reporter molecules the quantity of which is a proportiona measure of the amount of analyte in the specimen. For amplified assay procedures polymeric carrier molecules capable of binding multiple affinity liposomes and/or preformed complexes of affinity liposomes are utilized.

Activated Enzyme-Linked Detection Systems for Detecting and Quantifying Nucleid Acids, Antigens, Antibodies and Other Analytes

I. FIELD OF THE INVENTION

The present invention relates to assay procedures for detecting and quantifying analytes including nucleic acids, antigens, and antibodies using affinity liposomes capable of analyte-specific activation of solid phase-immobilized molecules for generating active reporter molecules. The detection precedures are preferably electrochemical or optical procedures.

II. BACKGROUND OF THE INVENTION

Numerous assays in the immunochemical and receptor-ligand fields have been developed for measuring virtually any biological molecule of clinical, biotechnological, and environmental importance. At present, the most frequently utilized methods are enzyme-linke immunosorbent assays (ELISAs). In conjunction with colorimetric, fluorescent, or chemiluminescent substrates these assays have gained wide acceptance for detecting substances such as antigens and antibodies in body fluids and tissues.

Conventional state of the art ELISA-type assays, however, pose problems if trace amounts of analytes have to be detected. Since only a limited number of enzyme molecules can be chemically attached or fused to detector molecules such as secondary antibodies, a high detection sensitivity requires generally a long analysis time due to extended enzymatic incubation periods. Furthermore, sensitive ELISA-type assays require the use of highly purified enzyme preparations for the synthesis of enzyme-detector molecule conjugates to avoid coupling of contaminating, enzymatically non-active proteins to detector molecules. Highly purified enzymes, however, are relatively expensive due to several labor-intensive purification steps.

In order to overcome some of these limitations, attempts have been made to increase the number of enzyme molecules per detector molecule via carrier systems. For practical purposes, however, the size of such polymeric carrier molecules has to be limited to avoid steric hindrance problems. Enzyme carriers which are too bulky are likely to affect the binding activity of chemically attached detector molecules such

as secondary antibodies. As a result, the number of enzyme molecules that may be chemically attached to suitable carrier molecules is limited.

Currently utilized molecular biology assays for detecting and quantifying nucleic acids in fields such as biotechnology, environmental protection, and public health use colorimetric, fluorescent, and chemiluminescent reporter systems. These labels have been linked or conjugated directly to nucleic acid reactants and products, or generated via nucleic acid-enzyme conjugates similar to ELISA techniques. Since these techniques have been borrowed from the immunochemical and receptor-ligand fields, they pose simlar problems with regard to detection sensitivity, time requirements, and cost effectivity as described for ELISA-type assays.

Recent advances in template amplification and signal amplification methods in conjunction with enzymatic detection procedures with colorimetric or chemiluminescent substrates have resulted in highly sensitive nucleic acid detection and quantitation techniques. For example, the branched chain DNA signal amplification technique has been demonstrated to provide sufficient sensitivity for monitoring human immunodeficiency virus type 1 burden in human plasma. These methods, however, are very expensive. Similar to highly sensitive ELISA-type assays, highly sensitive nucleic acid detection and quantitation techniques such as the branched chain DNA signal amplification assay require the use of highly purified enzyme preparations to avoid coupling of contaminating, enzymatically non-active proteins to detector oligonucleotides.

Attempts have been made to establish detection systems other than optical ones. Recently, the group of Prof. I. Willner, Israel, has shown an amplification method for electrical detection of DNA on electrodes. In this method, the interference of electrical impedance is amplified via use of large liposomes or nano-sized metal particles (lecture of April 1, 2000, at Analytica 2000, Munich, Germany).

Separation-free electrochemical detection procedures have been described by Duan, C., and Meyerhoff, M.E. (Anal. Chem. 66, 1369, 1994) and Meyerhoff, M., Duan, C., and Meusel, M. (Clin. Chem. 41, 1378, 1995). Their technique utilizes a gold-plated microporous membrane which serves as the solid phase for a non-competitive sandwich-type immunoassay as well as a working electrode of an amperometric detection system. A capture monoclonal antibody covalently immobilized at the gold-plated side of the membrane, is incubated with the analyte protein in the presence of an alkaline phosphatase-anti-analyte antibody conjugate. Surface-bound conjugate

is then detected by the introduction of an enzyme substrate such as 4-aminophenylphosphate from the backside of the membrane which is not gold-plated. The substrate seeps through the membrane and is converted by surface-bound alkaline phosphatase to electrochemically active 4-aminophenol. The current generated by oxidation of 4-aminophenol at the gold electrode is a measure of the concentration of analyte in the sample. The procedure avoids some of the disadvantages associated with optical ELISA-type assays, but the detection sensitivity is still too low despite an enzymatic amplification step. For example, the system described by Duan, C., and Meyerhoff, M.E. is only capable of detecting human chorionoic gonadotropin down to a level of 500 ng/L. An improved method for small-volume voltammetric detection of p-aminophenol by an interdigitated array micro-electrode cell has been reported by Niwa, O., Xu, Y., Halsall, H.B., Heineman, W.R. (Anal. Chem. 65, 1559, 1993). However, using the interdigitated array electrodes and alkaline phosphatase for amplification, the detection sensitivity of IgG antibodies was still in a range that is not viable for clinical applications with respect to the detection and quantitation of disease-specific antibodies.

The problems listed in the foregoing are not intended to be exhaustive, but rather to describe some of the factors that limit the value of the developed detection procedures. The enzymatic reporter systems utilized in these detection procedures require a novel design to provide a cost effective method for fast analyses with a satisfactory level of detection sensitivity. Therefore, despite of all recent attempts at improvement, there exists a need in the field for an alternative enzymatic assay for the detection and quantitation of analytes such as nucleic acids, antigens, antibodies, and other analytes.

III.SUMMARY OF THE INVENTION

It is the object of the present invention to provide a novel reporter system for the detection and quantitation of analytes, including preferably nucleic acids, antigens, and antibodies, but also low molecular weight substances like hormones, therapeutic drugs, and toxicological compounds. This reporter system substantially overcomes the limitations known in the prior art.

The invention provides systems (kits) and methods (assay procedures) for (i) selective binding of liposome-encapsulated enzyme activators to analytes bound to immobilized capture molecules by molecular biological or immunochemical mechanisms, and (ii) utilization of such enzyme activators for activation of solid phase-immobilized inactive enzyme molecules to generate reporter molecules for measurement and optionally for quantitation. The reporter molecules may be electrochemically detectable, the quantitation taking place via electrochemical methods, e.g. current producing redox processes (e.g. redox recycling). Alternatively, the reporter molecules may be optical detectable, the quantitation taking place via optical methods.

Thus, the novel detection system of the present invention may be designated as activated enzyme-linked detection system

In a first aspect of the invention, a reporter system for detecting analytes in a solution is provided, comprising the following components: A solid support containing immobilized capture molecules, a solid support containing immobilized inactive enzyme molecules, enzyme activator-containing affinity liposomes, and substrates for detection procedures. The substrates may be adapted for use in electrochemical detection. In this case, they are used as detectable species for an electrochemical sensor (transducer). Alternatively, the substrates can be tailored for colorimetric, fluorescent, chemiluminescent, or bioluminescent detection procedures. This system may be in the form of a kit.

Capture molecules include but are not limited to single-stranded ribo and deoxyribo nucleic acids, single-stranded ribo and deoxyribo oligonucleotides, 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogues, antigens, peptides antibodies, and other binding proteins such as receptor proteins. Analytes are bound to these capture molecules by molecular biological or immunochemical reactions. The affinity liposomes contain encapsulated enzyme activators and

surface-attached affinity components capable of specific binding to analytes, analyte derivatives, or analyte-reactive molecules, e.g. nucleic acids, antigens, antibodies, and derivatives thereof. Suitable nucleic acid-reactive affinity components include but are not limited to ribo and deoxyribo nucleic acids, ribo and deoxyribo oligonucleotides, 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogues, intercalating agents, intercalating agents conjugated to oligonucleotides or nucleic acids, and immunoglobulins or fragments of immunoglobulins with specificity for double- and/or triple-stranded nucleic acids. Other suitable affinity components include but are not limited to antigens, protein ligands, antibodies, antibody fragments, and other binding proteins. Enzyme activators include but are not limited to metals for restoration of the catalytic activity of inactive apometalloenzymes, protein fragments for complementation of inactive deletion mutant enzymes, and enzyme subunits for activation of incomplete enzyme complexes.

In a second aspect of the present invention, a method for detecting and optionally quantifying analytes in a liquid sample to be analyzed is provided, comprising the steps of (a) providing (i) capture molecules immobilized on the surface of a solid supportand capable of specifically binding said analyte, (ii) affinity liposomes containing encapsulated enzyme activator, the affinity liposomes or components bound or later to be bound thereto specifically binding to the analyte and/or capture molecules in case the analyte and the capture molecules are bound to each other, but not to free capture molecules, (iii) enzyme molecules in an inactive condition immobilized on the surface of the same solid support where the capture molecules are immobilized, or on a different support, and (iv) a substrate being convertible to reporter molecules via said enzyme molecules in activated condition, (b) contacting the sample with the immobilized capture molecules in such a way that analytes having the capability to bind to the capture molecules are bound thereto, (c) adding said enzyme activator-containing affinity liposomes, (d) removing unbound affinity liposomes, (e) at least partial lysing or otherwise opening said affinity liposomes, thereby releasing encapsulated enzyme activator from the interior thereof, (f) bringing the immobilized, inactive enzyme molecules into contact with enzyme activator released from the interior of said affinity liposomes and restoring the activity of said enzyme molecules, (g) adding the substrate being convertible into reporter molecules, and measuring the reporter molecules thereupon formed. The expression "lysing or opening affinity liposomes" means that the liposomal wall is at least permeable or porous in such a way that the interior comes into contact with the exterior and the content is at least partly released.

The components of the present invention allow the design of various assay configurations. Basic assay procedures include binding of affinity liposomes to captured analyte bound to capture molecules linked to a solid support, release of enzyme activators from analyte-bound affinity liposomes, activation of solid phase-immobilized inactive enzyme molecules by released enzyme activators, and generation of reporter molecules by activated enzyme molecules, and optical or electrical quantification of the thus generated optically or electrochemically detectable reporter molecules, e.g.via optical or electrochemical methods.. Depending on the lipid composition of affinity liposomes, the release of encapsulated enzyme activators from specifically bound affinity liposomes is effected e.g. by detergent-mediated lysis or by a moderate increase of the ambient temperature. The quantity of generated optically or electrochemically active reporter molecules is a proportional measure of the quantity of analyte in the specimen.

In a specific and preferred embodiment of the present invention, polymeric carrier molecules capable of binding multiple (i.e. more than one per molecule, preferably 2 to 50 and more preferred 5 to 20) affinity liposomes and/or preformed complexes of affinity liposomes may be utilized for amplified assay procedures. If the said polymeric carrier molecules are utilized, they can be designed to bind to the analyte and/or capture molecules in case the analyte and the capture molecules are bound to each other, but not to free capture molecules. In such cases, the polymeric carrier molecules are added to the capture molecules after contacting the capture molecules with the sample possibly containing the analyte to be detected, but before the enzyme activator-containing liposomes are added. Upon addition of the activator-containing liposomes (or complexes of such liposomes), a respective number of liposomes may be bound to each of the carrier molecules, thus providing amplification of binding events and thus of conversion of substrate to reporter molecules, yielding an amplification of measured signal. The same applies in case preformed complexes of affinity liposomes are used. In this case, one binding event of an affinity component of one liposome of said complex yields binding of a number of affinity liposomes identical with number of the affinity liposomes complexed to each other in the said complex. A further amplification may be obtained if both methods, the method of using polymeric carrier molecules, and the method of using preformed complexes of affinity liposomes, are used together. In this case, each of the polymeric carrier molecules bound to a structure of the capture molecule/analyte will be able to bind more than one (preferably 2 to 10) complexes of affinity liposomes. Thus, the present invention provides an enzymatic amplification system that allows signal enhancement by several orders of magnitude. Since affinity

liposomes allow encapsulation of large amounts of enzyme activators, multiple enzyme molecules are activated upon release of such enzyme activators from each analyte-bound affinity liposome.

The present invention provides an additional advantage in that only partially purified enzyme preparations may be utilized. The size of the solid phase area can be easily adjusted to accommodate additional contaminating, enzymatically inactive proteins. As a result, the present invention reduces costs and provides a novel technique for highly efficient signal amplification.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Detection of target nucleic acids using affinity liposomes containing encapsulated Zn^{2+} ions and surface-attached intercalating agents. Figure legend: solid support (1), optionally comprising a sensor surface; surface bound capture oligonucleotide (2); target DNA (3); region of DNA double helix (4); affinity liposome (5); intercalating residues (In) (6); flexible spacer molecules (7); immobilized, inactive apometallo enzymes (8).

Figure 2 Detection of high molecular weight antigens using affinity liposomes containing encapsulated Zn^{2+} ions and surface-attached antigen-specific antibodies. Figure legend: solid support (1), optionally comprising a sensor surface ; affinity liposome (5); flexible spacer molecules (7); immobilized, inactive apometallo enzymes (8); surface-bound anti-antigen A antibody I (9); antigen A (10); antigen B (11); antigen C (12); liposome-bound anti-antigen A antibody II (13).

V. DETAILED DESCRIPTION OF THE INVENTION

In its broadest aspect, the reporter technology of this invention is capable of detecting and quantifying analytes by immunochemical and molecular biological procedures for analytical and clinical applications. Examples of clinical applications include but are not limited to the analysis of nucleic acids or amplicons thereof, antigens, and antibodies in body fluids or tissues. Analyses of specific nucleic acids or nucleic acid sequences are commonly but not exclusively used to examine for the presence of infectious microorganisms, malignancies, inherited genetic defects, forensic medical evidence, pharmocogenomics, and paternity/maternity identification. Analyses of antigens and antibodies in clinical applications are commonly but not exclusively used for diagnosis of infectious diseases (e.g., microbial antigens or antibodies), autoimmune diseases (autoantigens or autoantibodies), malignancies (oncologic marker including tumor-specific proteins), and other diseases leading to cell death (e.g., myocardial infarction) and, thereby, to the release of cellular components into circulation. In addition, the reporter technology of this invention is also applicable for detecting and quantifying low molecular weight compounds such as hormones (e.g., polypeptides, steroids), therapeutic drugs, and toxicologic compounds.

If detection is intended to be an electrochemical detection, the system includes a transducer or electrochemical sensor, comprising e.g. a closely spaced (micrometer or submicrometer scale) electrode array (e.g. thin film nobel metal or carbon electrodes) for voltammetric (including amperometric) quantitation of electrochemically detectable reporter molecules (generated by immobilized activated enzyme molecules) via current producing redox processes (e.g., redox recycling), or the like. The immobilized activated enzyme molecules may be located at separate solid carriers or may be immobilized on the electrosurface which may be the said electrode array, e.g. between the electrodes and/or directly on the electrodes, or around the electrode arrays.

Capture molecules include but are not limited to single-stranded ribo and deoxyribo nucleic acids, single-stranded ribo and deoxyribo oligonucleotides, 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogues, antigens, antibodies, and other biologic recognition molecules including peptides, cell adhesion molecules, cell surface receptor molecules, solubilized (e.g., truncated) cell surface receptor molecules, intracellular receptor molecules (e.g., hormone binding proteins), and nucleic acid binding proteins (e.g., the prokaryotic lac repressor or the

eukaryotic cyclic AMP responsive element binding protein (CREB)). Non-biologic binding molecules such as "molecular imprints" (synthetic polymers with pre-determined specificity for binding or complex formation) are also applicable to the invention. Analytes are bound to these capture molecules by molecular biological or immunochemical reactions. For capturing of target nucleic acids or amplicons thereof, 'preorganized' oligonucleotide structures rigidified in a position that resembles the bound conformation, offer a potential advantage over single-stranded nucleic acids or single-stranded oligonucleotides since they exhibit a higher affinity for target nucleic acids.

The affinity liposomes contain encapsulated enzyme activators and surface-attached affinity components capable of specifically binding to said captured analytes and/or capture molecules in case the analyte and the capture molecules are bound to each other, but not to free capture molecules. Alternatively, the affinity components are capable in specific cases of specifically binding to structures which in turn are able to bind to said captured analytes and/or capture molecules in case the analyte and the capture molecules are bound to each other, but not to free capture molecules. Preferred nucleic acid-reactive affinity components include but are not limited to ribo and deoxyribo nucleic acids, ribo and deoxyribo oligonucleotides, 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogues, intercalating agents, intercalating agents conjugated to oligonucleotides or nucleic acids, and immunoglobulins or fragments of immunoglobulins with specificity for double- and/or triple-stranded nucleic acids. The nucleic acids and oligonucleotides are designed for hybridization to single-stranded segments of captured target nucleic acids or amplicons thereof. Suitable intercalating agents are selected from a group exhibiting a preference for double-stranded and/or triple-stranded nucleic acids including actinomycin D derivatives, anthracycline derivatives, acridine derivatives, cyanine dye derivatives, hydroxystilbamidine derivatives, imidazole derivatives, indole derivatives, phenanthridine derivatives, and psoralen derivatives. Preferred affinity components capable of specific binding to captured antigens, antibodies, and derivatives thereof include but are not limited to antigen-specific antibodies or antigen-specific antibody fragments, antigens, protein ligands, and other binding proteins. Preferred affinity components include also those which are capable of specifically binding to a complementary affinity component conjugated to a hapten molecule or to an analyte-reactive reagent such as a secondary antibody. Examples of affinity systems providing complementary affinity components include but are not limited to hapten / anti-hapten antibody systems, enzyme inhibitor / enzyme systems, and the biotin / (strept)avidin affinity system.

Preferred encapsulated enzyme activators include but are not limited to metals for restoration of the catalytic activity of inactive apometalloenzymes, protein fragments for complementation of inactive deletion mutant enzymes, and enzyme subunits for activation of incomplete enzyme complexes. Metalloenzymes suitable for this invention include, but are not limited to metallophosphatases (e.g., alkaline phosphatase from *Escherichia coli*), metallodehydrogenases (e.g., alcohol dehydrogenase from horse liver), and metalloproteases (e.g., aminopeptidase M from pig kidney). The intrinsic metal atoms of such metalloenzymes can be removed by treatment with appropriate chelating agents and replaced completely with corresponding loss and restoration of catalytic activity. Metals encapsulated in affinity liposomes may also be utilized as buffer constitutents to mediate catalytic activity of non-metalloenzymes which have an absolute requirement for certain metals. Examples of such enzymes include but are not limited to venom exonuclease (phosphodiesterase I) from *Crotalus adamanteus* which has an absolute requirement for Mg^{2+} and is completely inhibited by treatment with the chelating agent EDTA. Preferred protein fragments for complementation of inactive deletion mutant enzymes include but are not limited to polypeptides capable of activating inactive β-galactosidase deletion mutants. For example, the 90-residue peptide CB2 restores the activity of the inactive deletion M15 mutant (α-acceptor) of β-galactosidase from *Escherichia coli* to approximately two-thirds of the level of native β-galactosidase.

The enzymes used in the present invention immobilized on a support and in inactive condition may be selected from those enumerated above in connection with the respective enzyme acitvators.

Suitable substrates for activated enzyme molecules are selected conveniently in regard the enzyme used in the system and will differ in regard the detection system to be used.

If detection is intended to be an optical detection, said substrates will include but are not limited to phosphomonoester derivatives of reporter molecules for metallophosphatases, phosphodiester derivatives of reporter molecules for venom exonuclease, galactoside derivatives of reporter molecules for β-galactosidase and amino acid derivatives of reporter molecules for metalloaminopeptidases and metallocarboxypeptidases. Such substrates are utilized to generate colorimetric, fluorescent, chemiluminescent, or bioluminescent reporter molecules. Preferred examples of suitable substrates for colorimetric determinations include but are not

limited to 4-nitrophenyl-phosphate for alkaline phosphatase, p-nitrophenyl thymidine-5'-phosphate or bis(p-nitrophenyl) phosphate for venom exonuclease, hippuryl-L-phenylanine for carboxypeptidase A, hippuryl-L-arginine for carboxypeptidase B, and 2-nitrophenyl-β-D-galactoside for β-galactosidase. Preferred examples of suitable substrates for fluorimetric determinations include but are not limited to 4-methylumbelliferyl phosphate (4MUP) for alkaline phosphatase and 4-methylumbelliferyl-β-D-galactopyranoside (4MUG) for β-galactosidase. Preferred examples of suitable substrates for time-resolved fluorimetric determinations include but are not limited to 5-fluorosalicylic phosphate for alkaline phosphatase. The product of the enzymatic hydrolysis, 5-fluorosalicylate, forms a highly fluorescent complex with Tb^{3+}-EDTA in alkaline solution. Preferred examples of suitable substrates for chemiluminescent detection of alkaline phosphatase include but are not limited to adamantyl 1,2-dioxetane aryl phosphates such as disodium 3-(4-methoxyspirol [1,2-dioxetane-3,2'-tricyclo [3.3.1.1$^{3.7}$] decan-4-yl] phenyl-phosphate) (AMPPD) and the 5-chloro-substituted analog (CSPD). Alkaline phosphatase dephosphorylates these substrates to produce a phenoxide intermediate that generates light emission at 470 nm upon decomposition. Alternatively, activated alkaline phosphatase molecules may be quantitated by a chemiluminescent detection reaction using ascorbic acid-2-O-phosphate as substrate. Enzymatic cleavage of the phosphate group generates ascorbic acid which is quantitated under alkaline conditions in the presence of oxygen and lucigenin. Another chemiluminescent detection reaction of alkaline phosphatase utilizes glucose-1-phosphate as substrate. Enzymatically generated glucose molecules are further reacted with glucose oxidase and the resulting hydrogen peroxide is quantitated using a mixture of microperoxidase and luminol. A related chemiluminescent detection reaction of alkaline phosphatase utilizes 4-iodophenyl phosphate as substrate. Enzymatically generated 4-iodophenol is quantitated using horseradish peroxidase, hydrogen peroxide, and lunimol. Still another chemiluminescent detection reaction of alkaline phosphatase utilizes $NADP^+$ as substrate and the resulting NAD^+ is used as a cofactor by alcohol dehydrogenase which converts ethanol to acetaldehyde with the concomitant reduction of NAD^+ to NADH. In a subsequent reaction, NADH is quantitated using a mixture of microperoxidase, 1-methoxy-5-methylphenazinium methyl sulfate (1-MPMS), and luminol. Preferred examples of suitable substrates for chemiluminescent detection of activated _-galactosidase molecules include but are not limited to disodium 3-(4-methoxyspirol [1,2-dioxetane-3,2'-tricyclo [3.3.1.1$^{3.7}$] decan-4-yl] phenyl-galactoside) (AMPGD). Enzymatic cleavage of the galactoside group from the 3-position of the aromatic ring of AMPGD produces the phenoxide derivative described above. Another

chemiluminescent detection reaction of activated β-galactosidase molecules utilizes lactose. Enzymatically generated glucose molecules are further reacted with glucose oxidase and the resulting hydrogen peroxide is quantitated using a mixture of microperoxidase and luminol, or a mixture of 8-anilino-1-naphthalenesulfonic acid (ANS) and bis (2,4,6-trichlorophenyl) oxalate (TCPO). Preferred examples of suitable substrates for bioluminescent detection of activated enzyme molecules include but are not limited to D-luciferin derivatives such as D-luciferin-O-phosphate for alkaline phosphatase, D-luciferin-O-β-galactoside for β-galactosidase, D-luciferyl-L-Nα-arginine for detector reagents with carboxypeptidase B and N activities, and D-luciferyl-L-phenylalanine for detector reagents with carboxypeptidase A activity. Methods for quantification of released D-luciferin include but are not limited to luminometric detection assays using the bioluminescence system of *P. pyralis*.

Suitable quantification systems of activated enzyme molecules include single- and multi-stage assays as well as recycling assay systems. Preferred single- and multi-stage quantification assays include but are not limited to those described above. Preferred recycling systems for alkaline phosphatase include but are not limited to procedures in which $NADP^+$ (nicotinamide adenine dinucleotide phosphate) is converted to NAD^+ by alkaline phosphatase. NAD^+ is used as a cofactor by alcohol dehydrogenase which converts ethanol to acetaldehyde with the concomitant reduction of NAD^+ to NADH. Subsequently, diaphorase catalyzes the reduction of another compound, thereby generating a chromogenic, fluorescent, chemiluminescent, or bioluminescent reporter molecule. This reaction regenerates the NAD^+. Therefore, activated alkaline phosphatase molecules produce many NAD^+ molecules which are not consumed in the system but are recycled continuously, generating one reporter molecule per cycle. Useful chromogenic diaphorase substrates include but are not limited to p-iodonitrotetrazolium violet which forms an intensely purple formazan dye upon reduction. Useful fluorogenic diaphorase substrates include but are not limited to resazurin which forms the fluorescent compound resorufin upon reduction.

If detection is intended to be an electrochemical detection, those substrates are preferred which may be used in voltammetric, e.g. and preferable amperometric measurements, e.g. in redox processes, for example redox recycling. Said substrates will include but are not limited to phosphomonoester derivatives of redox mediators for metallophosphatases, phosphodiester derivatives of redox mediators for venom exonuclease, amino acid derivatives of redox mediators for metalloaminopeptidases, and galactoside derivatives of redox mediators for β-

galactosidase. Prior to enzymatic action, such derivatives are essentially electrochemically not detectable, while they are susceptible to redox reycling after enzymatic cleavage. Examples of redox mediators suitable for this kind of derivatization include but are not limited to aromatic ring structures substituted with appropriate organic residues that allow switching from a regular aromatic structure to a quinone structure and vice versa. The benzoquinone / hydroquinone couple and the p-aminophenol / quinoneimine couple are well-known redox couples of this class.

The said electrochemically detectable mediators (reporter molecules) generated by activated enzyme molecules are quantitated via voltammetric (current producing) and, more preferably, amperometric processes, e.g., redox processes, for example redox recycling. If voltammetry is selected for detection, mesasurement is performed in conjunction with a preferably closely spaced (micrometer and submicrometer scale) array of electrodes, e.g. thin film noble metal electrodes. Preferred are interdigitated arrays where anodes and cathodes have a width between 20 and about 800 nm and the electrodes are spaced apart from each other with a distance between 5 and 5000 nm. In a more preferred embodiment, the electrodes are spaced apart from each other with a distance between 100 and about 800 nm. In another preferred embodiment, the line the space ratio is 1:1 in order to obtain efficient redox recycling.

In either case, the quantity of generated reporter molecules is a proportional measure of the amount of analyte in the specimen, and thus, quantification of analyte is possible.

The basic assay procedure includes binding of the analyte to immobilized capture molecules either by immunochemical or molecular biological interactions, detection of captured analytes by analyte-specific binding of affinity liposomes containing encapsulated enzyme activators, removal of non-bound affinity liposomes, release of enzyme activators from specifically bound liposomes and utilization of such enzyme activators for activation of immobilized inactive enzyme molecules, generation of reporter molecules which are preferably optical active reporter molecules or, more preferred, electrochemically detectable reporter molecules via activated enzymes, and quantification of such reporter molecules by a detection system, preferably optical or electrochemical detection. The expression "analyte-specific binding of affinity liposomes" does not only mean binding of affinity liposomes to analyte bound to capture molecules, but also includes binding of affinity liposomes to structures of the capture molecules which are affected by the binding event with the analyte or to

structures of the capture molecule-analyte combination ("complex") thus formed (e.g. double stranded nucleic acid formed upon adding single stranded nucleic acid analyte to a capture molecule comprising single stranded nucleic acid) and binding to polymeric carriers which in turn are bound to affected capture molecule and/or bound analyte.

In one embodiment of the present invention, the novel activated enzyme-linked detection system is applied for detecting or quantifying specific nucleic acids or amplicons thereof. In preferred assay procedures, nucleic acids (or amplicons thereof) to be analyzed are captured by hybridization to immobilized single-stranded capture oligonucleotides (or 'preorganized' oligonucleotide structures) and detected with affinity liposomes carrying surface-attached nucleic acid-reactive affinity components **(Fig.1)**. The release of enzyme activators encapsulated in specifically bound affinity liposomes is effected e.g. by an increase of the ambient temperature (method A) or the addition of liposome lysing solvents (method B). The substrate can be added to the immobilized inactive enzymes with a preceding washing step (preferred for method A) or together with the liposome-lysing solvent (in method B). The quantity of enzymatically generated reporter molecules is a proportional measure of the amount of target nucleic acid (or amplicons thereof) in the specimen.

In another embodiment of the present invention, the novel activated enzyme-liked detection system is applied for detecting and quantifying antigens and antibodies. In preferred assay procedures, high molecular weight antigens (e.g., proteins and protein derivatives) to be analyzed are captured by binding to immobilized antibodies and detected in a sandwich-type assay procedure with affinity liposomes carrying surface-attached antibodies with specificity for the captured antigen **(Fig. 2)**. Similarly, antibodies to be analyzed are captured by binding to immobilized antigen molecules and detected with affinity liposomes carrying surface-attached antibodies with specificity for the captured antibody subclass. The release of enzyme activators encapsulated in specifically bound affinity liposomes is effected e.g. by an increase of the ambient temperature (method A) or the addition of liposome-lysing solvents (method B). The substrate can be added to the immobilized inactive enzymes with the preceding washing step (e.g. in method A) or together with the liposome-lysing solvent (in method B). The quantity of enzymatically generated reporter molecules is a proportional measure of the amount of antigen or antibody in the specimen.

In addition, the present invention allows for the detection and quantification of low molecular weight compounds (haptens) using hapten molecules labeled with affinity

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components which are capable of binding affinity liposomes containing surface-attached complementary affinity components or using hapten molecules bound to the surface of the affinity liposomes. Examples of affinity systems providing complementary affinity components include but are not limited to hapten / anti-hapten antibody systems, enzyme inhibitor / enzyme systems, and the biotin / (strept)avidin affinity system. In a first embodiment, the labeled hapten molecules are added in selectively limited quantities to samples containing the low molecular weight compound of interest. In such a format (competition assay), the quantity of labeled hapten complexed to the capture antibody will be dependent on the relative ratios of non-labeled and labeled hapten. Labeled hapten molecules captured by immobilized hapten-specific antibody are detected with affinity liposomes capable of specifically binding to the hapten-attached affinity component. In a second embodiment, the analyte containing sample is mixed with a limited amount of affinity liposomes carrying analyte molecules on their surface. The mixture is then brought into contact with proper capture molecules which are able to bind to the analyte. Binding of analyte from the sample is a competitive event with binding of analyte attached to the surface of affinity liposomes. The release of enzyme activators encapsulated in specifically bound affinity liposomes is effected e.g. by an increase of the ambient temperature (method A) or the addition of liposome-lysing solvents (method B). The substrate can be added to the immobilized inactive enzymes with the preceding washing step (e.g. in method A) or together with the liposome-lysing solvent (in method B). In the first embodiment described above, the quantity of enzymatically generated reporter molecules is a proportional measure of the amount of hapten in the specimen (sample).

Assay procedures including additional or modified steps are specific embodiments of the basic concept illustrated above.

In one preferred embodiment of the invention, additional amplification molecules are included in the assay procedure for signal amplification. In one amplified assay procedure, captured antigens, antibodies or target nucleic acids (or amplicons thereof) are detected by polymeric carrier molecules containing two different affinity components, one for specific binding to captured analytes and the other for binding of affinity liposomes. Preferred affinity components capable of specific binding to captured analytes are the same as those on the surface of affinity liposomes capable of specific binding to captured analytes. Preferred affinity systems for binding of affinity liposomes to polymeric carrier molecules include but are not limited to hapten / anti-hapten antibody affinity systems, enzyme inhibitor / enzyme affinity systems,

and the biotin / (strept)avidin affinity system. Since polymers allow covalent attachment of multiple affinity components, each captured analyte molecule can bind multiple affinity liposomes leading to an effective signal amplification. Preferred examples of synthetic and natural polymer derivatives include but are not limited to derivatives of polysaccharides, polyamino acids, polyvinyl alcohols, polyvinylpyrrolidinones, polyacrylic acids, various polyurethanes, polyphosphazenes, and copolymers of such polymers. In a more preferred embodiment, derivatives of dextran are employed as polymeric carrier molecules.

In one embodiment of the present invention utilizing such polymeric carrier molecules, captured analyte (e.g., target nucleic acids or amplicons thereof) is detected by dextran polymers containing two types of low molecular weight affinity components. One of the covalently linked affinity components is capable of specifically binding to the captured analyte (e.g., a specific captured target nucleic acid) in a structure restricted manner (e.g., intercalating agents or oligonucleotides). The other covalently linked affinity components (e.g., hapten molecules, enzyme inhibitors, or biotin residues) are capable of specifically binding to complementary proteinaceous affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) on the surface of affinity liposomes. The release of enzyme activators from affinity liposomes specifically bound to analyte-coupled dextran polymers and quantitation of reporter molecules generated by activated enzymes is performed as described.

In another embodiment of the present invention utilizing such polymeric carrier molecules, captured analyte is detected by dextran polymers containing two types of proteinaceous affinity components. One of the covalently linked affinity components is capable of specifically binding to the captured analyte in a structure restricted manner (e.g., antigen-specific antibodies, antibodies with specificity for the subclass of the captured antibody, or antibodies with specificity for double- and/or triple-stranded nucleic acids). The other covalently linked affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) are capable of specifically binding to complementary affinity components (e.g., hapten molecules, enzyme inhibitors, or biotin residues) on the surface of affinity liposomes. The release of enzyme activators from affinity liposomes specifically bound to analyte-coupled dextran polymers and quantitation of reporter molecules generated by activated enzymes is performed as described.

In a third embodiment of the present invention utilizing such polymeric carrier molecules, captured analyte is detected by dextran polymers containing proteinaceous affinity components derivatized with low molecular weight affinity components. If the proteinaceous affinity components are capable of specifically binding to the captured analyte in a structure restricted manner (e.g., antigen-specific antibodies, antibodies with specificity for the subclass of the captured antibody, or antibodies with specificity for double- and/or triple-stranded nucleic acids), the low molecular weight affinity components (e.g., hapten molecules, enzyme inhibitors, or biotin residues) are capable of specifically binding affinity liposomes containing surface-attached complementary proteinaceous affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) capable of binding to the low molecular weight affinity components on the dextran polymers. Vice versa, if the low molecular weight affinity components (e.g., intercalating agents or oligonucleotides) are capable of specifically binding to the captured analyte (e.g., a specific captured target nucleic acid) in a structure restricted manner, the proteinaceous affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) are capable of specifically binding affinity liposomes containing surface-attached complementary low molecular weight affinity components (e.g., hapten molecules, enzyme inhibitors, or biotin residues) capable of binding to the proteinaceous affinity components on the dextran polymers. The release of enzyme activators from specifically bound affinity liposomes and quantitation of reporter molecules generated by activated enzymes is performed as described.

In another preferred embodiment of the present invention, signal amplification is achieved by using preformed complexes of affinity liposomes. One preferred method for the preparation of complexed affinity liposomes utilizes affinity liposomes containing two types of surface-attached affinity components, one for specific binding to captured analytes and the other for complexation of affinity liposomes via bridging molecules. Preferred affinity components capable of specific binding to captured analytes including nucleic acids, antigens, antibodies, and derivatives thereof, are the same as those on the surface of non-complexed affinity liposomes capable of specific binding to captured analytes. Preferred affinity components for complexation of affinity liposomes via bridging molecules include but are not limited to hapten molecules, enzyme inhibitors, and biotin residues. Suitable bridging molecules are selected from a group including a) bi- or oligovalent anti-hapten antibodies or fragments thereof, as well as conjugates or fusion constructs thereof; b) enzymes, enzyme conjugates, and fusion constructs of enzymes providing more than one inhibitor-binding site; and c) avidin and streptavidin. Any bridging molecule

that provides more than one binding site is useful for this type of amplification methodology.

A first group of preformed complexes of affinity liposomes prepared via bridging molecules include but are not limited to complexes generated by reaction of i) (strept)avidin with affinity liposomes containing surface-attached biotin residues and surface-attached low molecular weight affinity components (e.g., low molecular weight nucleic acid-reactive components such as intercalating agents or oligonucleotides), ii) anti-hapten antibody molecules providing more than one hapten-binding site with affinity liposomes containing surface-attached hapten molecules and surface-attached low molecular weight affinity components (e.g., low molecular weight nucleic acid-reactive components such as intercalating agents or oligonucleotides), and iii) enzyme conjugates providing more than one inhibitor-binding site with affinity liposomes containing surface-attached inhibitor molecules and surface-attached low molecular weight affinity components (e.g., low molecular weight nucleic acid-reactive components such as intercalating agents or oligonucleotides).

Another group of preformed complexes of affinity liposomes prepared via bridging molecules include also complexes generated by reaction of i) (strept)avidin with affinity liposomes containing surface-attached proteinaceous affinity components (e.g., antigen-specific antibodies for binding to captured antigens, high molecular weight antigens for binding to captured antibodies, antibodies with specificity for the subclass of captured antibodies, or antibodies with specificity for double- and/or triple-stranded nucleic acids) derivatized with biotin residues, ii) anti-hapten antibody molecules providing more than one hapten-binding site with affinity liposomes containing surface-attached proteinaceous affinity components (e.g., antigen-specific antibodies for binding to captured antigens, high molecular weight antigens for binding to captured antibodies, antibodies with specificity for the subclass of captured antibodies, or antibodies with specificity for double- and/or triple-stranded nucleic acids) derivatized with hapten molecules, and iii) enzyme conjugates providing more than one inhibitor-binding site with affinity liposomes containing surface-attached inhibitor molecules and surface-attached (e.g., antigen-specific antibodies for binding to captured antigens, high molecular weight antigens for binding to captured antibodies, antibodies with specificity for the subclass of captured antibodies, or antibodies with specificity for double- and/or triple-stranded nucleic acids) derivatized with enzyme inhibitors.

For the preparation of a third group of preformed complexes of affinity liposomes, polymeric carrier molecules containing covalently coupled affinity components are employed as bridging molecules. The application of polymeric carrier molecules for complexation of affinity liposomes is useful when affinity components are utilized as bridging molecules which contain only a single binding site for the correponding affinity partner (e.g., anti-hapten single-chain antibodies (scFv) and enzymes containing only a single inhibitor binding site).

In still another embodiment, complexed affinity liposomes are prepared utilizing two types of affinity liposomes containing different affinity components. The affinity components on type I affinity liposomes are capable of specifically binding to a specific captured analyte (e.g., single-stranded oligonucleotides complementary to single-stranded segments of captured target nucleic acid or amplicons thereof, or antibodies with specificity for captured antigens). The affinity components on type II affinity liposomes are capable of specifically binding to the affinity components on type I affinity liposomes (e.g., single-stranded oligonucleotides complementary to type I single-stranded oligonucleotides, or antibodies with specificity for the subclass of the antibody on type I affinity liposomes).

In case the assay procedure (detection of analytes) utilizes preformed complexes of affinity liposomes, analyte may be bound to immobilized capture molecules e.g. by immunological or molecular biological interactions. Detected is performed by specific binding of preformed complexes of affinity liposomes containing surface-attached analyte-reactive affinity components. The release of enzyme activators from specifically bound complexes of affinity liposomes and quantitation of reporter molecules generated by activated enzymes is performed as described.

In another embodiment of the amplification assay procedure, signal amplification is achieved by a combination of polymeric carrier systems and preformed complexes of affinity liposomes. In one preferred amplified assay procedure utilizing a combination of polymeric carrier systems and preformed complexes of affinity liposomes, captured analyte is detected by dextran polymers containing two types of covalently linked affinity components, one being capable of specifically binding to the captured analyte, and the other being capable of specifically binding preformed complexes of affinity liposomes. The release of enzyme activators from specifically bound complexes of affinity liposomes and quantitation of reporter molecules generated by activated enzymes is performed as described.

Thus, the present invention provides an enzymatic amplification system that allows signal enhancement by several orders of magnitude. Since affinity liposomes allow encapsulation of large amounts of enzyme activators, a multiplicity of enzyme molecules are activated by a few binding events. Although metal-protein dissociation constants are generally (but not always) below 10 nM, large amounts of apometallo-enzymes can be immobilized to ensure maximum association of added metal ions. The size of the solid phase area available for immobilization of inactive apoenzyme molecules can be easily adjusted to accommodate sufficient amounts of enzyme. For example, using alkaline phosphatase from *E. coli* which has a molecular weight of approximately 89 kD, an immobilization area of 25 nm^2 per enzyme molecule may be assumed. Based on this calculation, 1 mm^2 accommodates approximately 4 x 10^{10} enzyme molecules corresponding to 6 ng of alkaline phosphatase. 6 ng (approximately 68 fmol) in 1 µl yields a concentration of 68 nm. Theoretically, a single binding event may be detectable. One small affinity liposome provides more than 10^5 molecules of low molecular weight enzyme activators such as Zn^{2+} which may activate up to 10^4 apoenzyme molecules of alkaline phosphatase from *E. coli*. Thereby, a single binding event may be amplified by more than eight orders of magnitude taking the high turn over number of alkaline phosphatase into account. Furthermore, the signal provided by a single analyte-bound affinity liposome can be easily amplified by one to two orders of magnitude if preformed complexes of affinity liposomes are used for detection.

Using a silicon microchip-formated array of closely spaced thin film nobel metal electrodes for electrochemical quantitation of enzymatically generated active redox mediators, a volume of only one microliter or even less is required for the amperometric detection. Consequently, the liposome-encapsulated enzyme activators can be released into a very small volume resulting in a relatively high concentration of enzyme activator which favors the activation process of inactive enzyme molecules. Using the same example as above, the release of 10^5 Zn^{2+} ions from a single affinity liposome into a detection volume of 2 µl will create a concentration of approximately 0.1 pM Zn^{2+}.

Since the size of the solid phase area can be adjusted according to the requirements of individual assay procedures, the present invention provides an additional advantage in that only partially purified enzyme preparations may be utilized for signal amplification. The presence of contaminating, non-enzymatic proteins can be tolerated provided sufficient space is available for immobilization. As a result, the

present invention reduces costs and provides a novel technique for highly efficient signal amplification.

The reporter system as described above may be provided in the form of a kit, the components of which are (a) a solid support containing the said immobilized capture molecules, (b) the affinity liposomes containing at least one surface-attached affinity component which comprise the said enzyme activator encapsulated therein, (c) the solid support carrying the said inactivated enzyme molecules, (d) the substrate convertible by activated enzyme molecules, and (e) optionally the said electrochemical sensor („transducer).

A specific arrangement is not required because measurement of the reporter molecules is independent from the immediate presence of the capture molecules. It is one of the advantages of the present invention that contacting the sample to be measured for the presence of analyte may be performed independent of performing the other steps of the inventive method, as time and/or area of performing the single method steps are concerned, provided that the components are sufficiently stable. Thus, measurement of the substrate which has been converted into reporter molecules by the activated enzyme may be performed in/on the same or in another vessel or plate (e.g. microtiter plate) in which the analyte had been bound to the capture molecules, and also possibly within a time limit which is suitably selected. It is possible that if an electrochemical sensor is present, the sensor is on the same support which carries the immobilized capture molecules or which carries the inactivated enzyme. Thus, the capture molecules or, alternatively, the inactivated enzyme molecules may be immobilized in the space between single electrodes. Alternatively, the support carrying the immobilized capture molecules is provided on a flat substrate which may be inserted into the container in which the (electrochemical or not electrochemical) detection is intended to be made. Further, the solid support carrying the said immobilized capture molecules and the solid support carrying the inactivated enzyme molecules may be the same support or different supports. In a preferred embodiment, the solid support containing immobilized capture molecules is at least a part or structure of the container or vessel In which the detection or a part thereof shall be performed. For example, the solid support is part of a variety of wells in a plate (e.g. microtiter plate), or is part of the bottom or the walls of the container or microtiter plate. The support carrying the immobilized enzyme molecules, if not the same support as the support carrying the capture molecules, may be in a fixed position relative to the latter. In another embodiment of the invention, the support carrying the immobilized enzyme

molecules is provided having the form of beads or a stripe or bar. In this case, it is preferred that the container intended for the detection comprises the immobilized capture molecules. This embodiment may also be provided vice versa, i.e. with capture molecules immobilized on beads or the like, the immobilized enzyme molecules being on a flat substrate, e.g. a structure of the wall or bottom of the vessel or microtiter plate in which the detection shall take place. Of course, it is also possible to provide supports (a) and (c) having the shape of beads or the like, in which case at least the beads or the like carrying capture molecules are added to the fluid containing the analyte.

If the support carrying the immobilized enzyme molecules is not part of the container, it may be added before or after the addition of the affinity liposomes. Care should be taken that the affinity liposomes bound to capture molecules remain wet or submersed in the liquid als long as they shall be kept intact. In this way, release of enzyme activator therefrom may be deferred. Since the enzyme activator is released from the affinity liposomes in the presence of analyte to be detected independent of whether the solid support carrying the inactive enzyme is present or not, it is possible to bring the analyte containing solution into contact with the immobilized capture molecules and to add the liposomes in a first step, and to convert the solution obtained upon release of the enzyme activator into a separate container where it is contacted with the immobilized enzyme molecules and with the substrate which in turn is converted into reporter molecules by activated enzyme. Hence, the method of the present invention results in the release of enzyme activation caused by the presence of analyte to be detected, while detection of the said activated enzyme may be performed separately (in regard to place and time). Alternatively, all steps to be performed in the present method may be performed in only one vessel and/or in an immediate sequence.

Subsequently, the components of the present invention are described in more detail.

V.1. ENZYMES AND ENZYME ACTIVATORS

In one embodiment of the invention, enzymes which require metal ions for enzymatic activity are employed for signal amplification. The metal atoms of such enzymes can be removed by treatment with appropriate chelating agents and replaced completely with corresponding loss and restoration of catalytic activity. Consequently, these enzymes can be immobilized as inactive metal-free molecules and activated by release of appropriate metal ions from specifically bound affinity-liposomes.

Metal binding enzymes are divided into two classes, metal-activated enzymes which associate metal ions losely (K_d = 10^{-3} to 10^{-8} M) and metalloenzymes which bind metal ions tightly (K_d <10^{-8} M). Examples of metal-activated enzymes include but are not limited to venom exonuclease (phosphodiesterase I) from *Crotalus adamanteus* which has an absolute requirement for Mg^{2+} and is completely inhibited by treatment with the chelating agent EDTA. **Table I** below, derived from the literature, gives examples of metalloenzymes from which inactive apoenzymes have been prepared.

Table I

Enzyme	Number of subunits	Metal/subunit	Chelating agent used to prepare apoenzyme
Alkaline Phosphatase (*E. coli*)	2	2 Zn^{2+}, 1 Mg^{2+}	1,10-phenanthroline, EDTA, Chelex 100
Aminopeptidase (*Aeromonas*)	1	2 Zn^{2+}	1,10-phenanthroline
Aminopeptidase (bovine lens)	6	2 Zn^{2+}	1,10-phenanthroline
Carboxypeptidase A	1	1 Zn^{2+}	1,10-phenanthroline
Procarboxypeptidase A	1	1 Zn^{2+}	1,10-phenanthroline
Carboxypeptidase B	1	1 Zn^{2+}	1,10-phenanthroline
Carboxypeptidase (*Steptomyces griseus*)	1	1 Zn^{2+}	1,10-phenanthroline
Neutral protease (*Bacillus subtilis*)	1	1 Zn^{2+}	EDTA
Thermolysin	1	1 Zn^{2+}, 1 Ca^{2+}	1,10-phenanthroline
Angiotensin converting enzyme	1	1 Zn^{2+}	1,10- phenanthroline
Alcohol dehydrogenase (horse liver)	2	2 Zn^{2+}	dipicolinic acid
Carbonate dehydratase	1	1 Zn^{2+}	1,10-phenanthroline, dipicolinic acid
Superoxide dismutase	2	2 Cu^{2+},1 Zn^{2+}	EDTA

Preferred metalloenzymes suitable for this invention include but are not limited to metallophosphatases (e.g., alkaline phosphatase from *Escherichia coli*), metalloproteases (e.g., aminopeptidases), and metallodehydrogenases (e.g., alcohol dehydrogenase).

Preferred methods to remove metal ions from metalloproteins employ metal-chelating agents. Removal of metal ions at neutral pH is preferred as most metalloproteins denature at extremes of pH. Apoalkaline phosphatase is a notable exception as it can be prepared from the the native enzyme by dialysis against 2 M

$(NH_4)_2SO_4$ at pH 9.0. In a more preferred embodiment, apoalkaline phosphastase from *E. coli* is obtained by overnight incubation of the native enzyme in the presence of 50 mM EDTA at pH 6.5 or by treatment with Chelex 100 at pH 8.0 (Chappelet-Tordo, D.et al., Biochemistry 13, 3754, 1974). The metal-free apoalkaline phosphatase is completely inactive. Active metallophosphatase can be obtained by reconstitution on addition of a five-fold molar excess of metal ion to the apoenzyme at pH 7.8.

It has been shown above that the apoenzyme may easily provided at a reasonably large concentration.

Preferred encapsulated enzyme activators include also polypeptides for complementation of inactive deletion mutant enzymes. Examples include, but are not limited to complementation of the inactive deletion mutant M15 (α-acceptor) of β-galactosidase from *Escherichia coli* by the 90-residue peptide CB2 which restores the activity of the M15 mutant to approximately two-thirds of the level of native β-galactosidase.

Also applicable for use with the current invention are enzyme complexes which require the presence of subunits for catalytic activity. Vice versa, liposome-encapsulated inhibitory subunits of enzymes may be utilized to inhibit immobilized active enzymes in a modified assay procedure in which a decrease in enzymatic activity is used as detection principle.

V.2. IMMOBILIZATION OF INACTIVE ENZYME MOLECULES

The solid support serving for immobilization of inactive enzyme molecules includes but is not limited to glassy or polymeric beads, microtiter plates and walls of the fluid containers used in the present detection system, metallic, porous, impervious or fibrous matrices or membranes or the like, as is well-known in the art. Such matrices may be utilized as derivatized (e.g., by adsorption of polylysine, phenylalanine-lysine, or octadecylamine) or non-derivatized solid supports for immobilization of inactive enzyme molecules. Immobilization of inactive enzyme molecules or derivatives thereof may be accomplished non-covalently or covalently by any of the well-known chemical coupling methods. Since the non-specific adsorption process affects the reproducibility, for most applications covalent immobilization techniques are preferred. Non-covalent, non-adsorptive immobilization techniques, however, are also applicable for this invention.

Preferred support matrices for immobilization of enzymes include but are not limited to agar, agarose, and Sephadex supports, cellulose, acrylic copolymers, polyacrylic and polymethacrylic copolymers, hydroxylalkyl methacrylate gels, nylon, anionic carriers obtained from nitrated copolymers of methacrylic acid with methacrylic acid-3-fluoroanilide or fluorostyrenes, copolymers of methacrylic acid and acrylic acid with 3- or 4-isothiocyanatostyrene, or copolymers of N-vinylpyrrolidone with maleic acid anhydride, derivatives of poly (vinyl alcohol), poly (allyl alcohol), or vinylether copolymers, and inorganic support materials such as ceramic and controlled-pore glass (for a review, see Weetall, H.H. Meth. Enzymol. 44, 134, 1976).

Hydrophilicity is a very important factor for the preservation of structural characteristics of enzymes after immobilization onto solid supports. This fact places hydrophilic supports in a favorable position among immobilized enzyme matrices. In the follwing examples, therefore, apometalloenzymes are immobilized onto cross-linked agarose beads using some of the many applicable immobilization techniques.

In one embodiment, inactive enzyme molecules are coupled via their primary amine groups to amine-reactive solid supports (e.g., N-hydroxysuccinimide ester-derivatized or aldehyde-derivatized solid supports). In another embodiment, inactive enzyme molecules containing a free sulfhydryl group are coupled to solid supports derivatized with sulfhydryl-reactive residues such as pyridyl disulfides, maleimides, or iodoacetyl groups. In still another preferred embodiment, suitable cross-linking reagents such as carbodiimides, homo-, or heterobifunctional cross-linkers are used for covalent immobilization of inactive enzyme molecules to functionalized solid supports. For example, inactive enzyme molecules may be derivatized with pyridyl disulfide groups (e.g., by reaction with N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)) and subsequently coupled to sulfhydryl-containing solid supports via disulfide linkages. Alternatively, the introduced sulfhydryl residues may be reduced with disulfide reducing agents to create terminal sulfhydryl groups for coupling to sulfhydryl-reactive solid supports. If SPDP should affect the activity of one of the enzymes, there are a number of additional cross-linking reagents for coupling via disulfide bonds such as 2-iminothiolane (2-IT) and N-succinimidyl S-acetylthioacetate (SATA). 2-IT reacts with primary amines, instantly incorporating an unprotected sulfhydryl group. SATA also reacts with primary amines, but incorporates a protected sulfhydryl group, which is later deacetylated using neutral hydroxylamine to produce a free sulfhydryl group. Other cross-linkers are available that can be used in different strategies for covalent coupling of inactive enzyme molecules to solid supports. S-(2-thiopyridyl)-L-cysteine hydrazide (TPCH) and S-(2-thiopyridyl)

mercaptopropiono hydrazide (TPMPH) react with the carbohydrate moieties of glycosylated enzymes that have been previously oxidized by mild periodate treatment, thus forming a hydrazone bond between the hydrazide portion of the cross-linking reagent and the periodate-generated aldehydes. TPCH and TPMPH introduce pyridyl disulfide residues which can be used for coupling as described above. If disulfide bonding is unfavorable, other cross-linking reagents may be used. For example, N-(γ-maleimido butyryloxy) succinimide (GMBS) and succinimidyl 4-(N-maleimidomethyl) cyclohexane (SMCC) react with primary amines, thereby introducing a maleimide group for coupling to sulfhydryl-containing solid supports via stable thioether linkages. Furthermore, cross-linking reagents may be used which introduce long spacer arms if steric hindrance problems interfere with the activity of covalently coupled enzyme molecules.

V.3. ENZYME SUBSTRATES

Suitable substrates for activated enzyme molecules may be selected depending on the detection method to be used.

V.3.1. Colorimetric substrates

Colorimetric substrates have been mentioned in general above.

In a more preferred embodiment, the limited dynamic range of colorimetric measurements is extended by simultaneous monitoring of absorbance at two or more wavelengths. In colorimetric assay procedures, the intensity of monochromatic light not adsorbed by the sample is measured. In order to quantitate low concentrations of chromogenic substrates, small differences in intensity have to be measured at high light intensity, a fact that limits the lower detection limit. Furthermore, the relationship between optical absorbance and the intensity of transmitted light is logarithmic. Therefore, at high concentrations of chromogenic substrates relatively large differences in optical absorbance correspond to very small differences in the intensity of unabsorbed light, limiting the precision and range of measurements. This limited dynamic range of colorimetric measurements, however, can be extended by simultaneous monitoring of absorbance at two or more wavelengths. For example, p-nitrophenol formed by alkaline phosphatase from p-nitrophenyl phosphate absorbs maximally at 405 nm. At 450 nm the molar extinction coefficient is about 20% of that at 405 nm, so that dual measurements at these wavelengths allows extension of the range of enzymatic activity readings by about a factor of five.

V.3.2. Fluorescent substrates

In another preferred embodiment, fluorescent substrates are employed for determining the quantity of activated enzyme molecules. The use of fluorescent substrates offers a dual advantage over chromogenic substrates in that the sensitivity of enzyme detection is significantly improved and the fluorimetric detection affords a wider assay dynamic range than spectrophotometric detection.

Preferably, interference from contaminating fluorescent compounds is reduced by time-resolved fluorimetric measurements. Preferred examples of suitable substrates for time-resolved fluorimetric determinations include but are not limited to 5-fluorosalicyl phosphate for alkaline phosphatase. The product of the enzymatic hydrolysis, 5-fluorosalicylate, forms a highly fluorescent complex with Tb^{3+}-EDTA in alkaline solution. The ternary fluorescent complex absorbs at a wavelength that is characteristic of the chelator (337 nm) and emits fluorescence with a long fluorescence lifetime that is characteristic of Tb^{3+}.

V.3.3. Chemiluminescent substrates

Alternatively, chemiluminescent substrates are employed for determining the quantity of activated enzyme molecules. Preferred examples of suitable substrates for chemiluminescent detection of alkaline phosphatase have been mentioned above. Examples are AMPPD and CSPD which are dephosphorylated by alkaline phosphatase dephosphorylates to produce a phenoxide intermediate that generates light emission at 470 nm upon decomposition. The light emission from this reaction is a long lived glow (>1 hr) and can be enhanced by certain polymers (e.g., polyvinylbenzyl (benzyldimethylammonium) chloride) and by detergent-fluorescein mixtures (Schaap, A.P., Akhaven, J., and Romano, L.J. Clin. Chem. 35, 1863, 1989). Alternatively, activated alkaline phosphatase molecules may be quantitated by other chemiluminescent detection reactions as mentioned above.

Preferred examples of suitable substrates for chemiluminescent detection of activated β-galactosidase molecules have also been mentioned above.

V.3.4. Bioluminescent substrates

In another preferred embodiment, bioluminescent substrates are employed for determining the quantity of activated enzyme molecules. Light production by firefly luciferase (bioluminescence of *P. pyralis*) occurs via oxidation of D-luciferin in the presence of ATP and Mg^{2+} to oxyluciferin with emission of light at 546 nm. The mechanism for light emission by bacteria (bacterial bioluminescence) requires

generation of NADH and subsequent reduction of flavin mononucleotide (FMN) to $FMNH_2$ which is the necessary cofactor for bacterial luciferase-mediated emission of light. In renilla bioluminescence, the luciferase substrate is a complex heterocyclic organic compound. Oxidation of renilla luciferin by renilla luciferase leads to carbon dioxide release. A high-energy intermediate is formed which is converted to oxyluciferin with concomitant light emission. Calcium-triggered luminescence of aequorin produces the blue-fluorescent protein (BFP). The protein part of BFP regenerates aequorin upon aerobic incubation with coelenterazine.

Preferred examples of suitable substrates for bioluminescent detection of activated enzyme molecules have also been mentioned above.

V.3.5. Recycling assay systems

In another embodiment of the present invention using optical detection systems, recycling assay systems are employed for determining the quantity of activated enzyme molecules. Preferred recycling systems for alkaline phosphatase include but are not limited to procedures in which $NADP^+$ (nicotinamide adenine dinucleotide phosphate) is converted to NAD^+ by alkaline phosphatase. NAD^+ is used as a cofactor by alcohol dehydrogenase which converts ethanol to acetaldehyde with the concomitant reduction of NAD^+ to NADH. Subsequently, diaphorase catalyzes the reduction of another compound, thereby generating a chromogenic, fluorescent, chemiluminescent, or bioluminescent reporter molecule. This reaction regenerates the NAD^+. Therefore, activated alkaline phosphatase molecules produce many NAD^+ molecules which are not consumed in the system but are recycled continuously, generating one reporter molecule per cycle. Useful chromogenic diaphorase substrates include but are not limited to p-iodonitrotetrazolium violet which forms an intensely purple formazan dye upon reduction. Useful fluorogenic diaphorase substrates include but are not limited to resazurin which forms the fluorescent compound resorufin upon reduction.

V.3.6 Substrates to be used in electrochemical detection systems

In principle, all those substrates for activated enzyme molecules may be used as reporter molecules which are capable to be oxidated or to be reduced, e.g. hydrochinones or napthols, organometals like complexes of osmium, ruthenium, cobalt or the like. More preferred (due to enhanced sensitivity) are those which may undergo redox recycling. Examples are phosphomonoester derivatives of redox mediators for metallophosphatases, phosphodiester derivatives of redox mediators

for venom exonuclease, amino acid derivatives of redox mediators for metalloaminopeptidases, and galactoside derivatives of redox mediators for β-galactosidase. Prior to enzymatic action, preferred derivatives are essentially electrochemically not detectable, while they are susceptible to redox reycling in arrays of closely spaced thin film nobel metal electrodes after enzymatic cleavage. Therefore, useful redox mediators have structures that can be chemically derivatized to generate substrates with the desired properties. One representative example of preferred redox mediators is *p*-aminophenol which has been adjusted for the alkaline phosphatase amplification system by converting the reporter molecule to the electrochemically inactive *p*-aminophenylphosphate (Risphon, J. et al., In: Biosensor Design and Application (P.R. Mathewson, and J.W. Finley, (eds.), pp. 59-70, Symposium Series 511, American Chemical Society, Washington, DC, USA, 1992).

Preferred redox mediators of this invention are further characterized by several additional important properties. Both the electrochemically not detectable substrate and the electrochemically detectable redox mediators should possess sufficient solubility in aqueous media to allow for a high substrate concentration and a good accessibility of generated redox mediator molecules to the microelectrodes. The redox potentials should exhibit reversible peaks in cyclic voltammograms and in aqueous solutions the potentials should be within the limits of - 600 mV (generation of oxygen) and + 800 mV (generation of hydrogen). Preferred are potentials close to 0 mV since they guarantee minimal background current as well as minmal instrumental background noise of the electrode, thereby providing optimal signal-to-noise ratios. Potentials close to 0 mV provide an additional advantage in that they minimize or avoid interference by electroactive compounds present in samples to be analyzed such as ascorbic acid in blood or catecholamines in urine.

One preferred class of redox mediators contains one or more aromatic ring structures and various organic substituents and side chains that allow switching from a regular aromatic ring structure to a quinone structure and vice versa. Some examples of suitable redox mediators and their electrochemical properties are listed in Table II.

Table II. Examples of suitable redox mediators

Mediator	E_{anodic} [mV]	$E_{cathodic}$ [mV]	Collection Efficiency	Detection limit [nM]
p-aminophenol	350	- 150	0.85	5 - 10
o-hydroquinone	600	- 200	0.92	5 - 10
o-benzoquinone	600	- 200	0.92	5 - 10
dopamine	800	- 100	0.88	20
aderenalin	400	- 50	0.86	50
1-naphthol	400	0	0.90	5 - 10

The benzoquinone/hydoquinone couple and the *p*-aminophenol/quinoneimine couple are well known redox couples of this class. Other preferred examples of this class of redox mediators include but are not limited to catechol and catechol derivatives (e.g., adrenalin, dihydroxyphenylalanine, epinine, adrenalone, norhomoepinephrine, and protocatechnic acid), dopamine, methoxytyraminin, aromatic compounds with more than one aromatic ring structure such as naphthol and anthracene derivatives, as well as heterocyclic aromatic compounds such as serotonin and hydroxyindolacetic acid. Aromatic redox mediators may be further derivatized with appropriate substituents to generate optimal redox potentials.

V.4. CAPTURE MOLECULES

As mentioned before, the invention comprises capture molecules for specific binding of analytes by molecular biological or immunochemical reactions. These molecules are immobilized on a solid support. Preferred capture molecules include but are not limited to antigens, antibodies, single-chain nucleic acids (ribo and deoxyribo nucleic acids), single-chain oligonucleotides (ribo and deoxyribo oligonucleotides), and 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogues. The term "antigen" refers to substances which can be recognized by in vitro or in vivo immune elements. Preferred, non-limiting examples of antigens include low molecular weight haptens such as steroids as well as high molecular weight antigens such as proteins.

The term "antibody" refers to immunoglobulins of any isotype or subclass as well as any fragment (e.g., Fab' of Fv fragments) of the aforementioned. Antibodies of any source are applicable including polyclonal materials obtained from any animal

species, monoclonal antibodies from any hybridoma source, and all immunoglobulins (or fragments) generated with the aid of viral, prokaryotic or eukaryotic expression systems.

Biologic recognition molecules other than antibodies are equally applicable for use with the current invention. These include but are not limited to peptides, cell adhesion molecules, cell surface receptor molecules, solubilized (e.g., truncated) cell surface receptor molecules, intracellular receptor molecules (e.g., hormone binding proteins), and nucleic acid binding proteins (e.g., the prokaryotic lac repressor or the eukaryotic cyclic AMP responsive element binding protein (CREB)). Non-biologic binding molecules such as "molecular imprints" (synthetic polymers with pre-determined specificity for binding or complex formation) are also applicable to the invention.

Single-chain nucleic acids and single-chain oligonucleotides are used to capture target nucleic acids or amplicons thereof by the formation of specific helical complexes. In a more preferred embodiment, 'preorganized' oligonucleotide structures are used as capture oligonucleotides (for a review, see Kool, E.T. Chem. Rev. 97, 1473, 1997). The rational for using 'preorganized' oligonucleotide structures is based on the observation that the affinity of oligonucleotides for the target nucleic acid can be increased by modifications rigidifying the oligonucleotide prior to binding so that it more resembles the bound conformation. An oligonucleotide rigidly held in the binding position prior to complexation shows less free internal bond rotations that need to be 'frozen' during complexation. At the same time, the molecule is organized into a shape which is more complementary to the desired target than to undesired ones. This increases selectivity because mismatched targets will cause unfavorable responses such as non-optimum bond angles or streric clashes.

Strategies for the construction of 'preorganized' oligonucleotide structures include i) enhancement of base stacking, ii) limitation of bond rotations, and iii) linking of binding domains. Double-, triple-, and quadruple-stranded nucleic acid helices are stabilized by base stacking and hydrogen bonding interactions. Since the majority of the base-stacking interaction in nucleic acids is between bases within a strand, the strengthening of stacking will have the tendency to cause single-stranded oligonucleotides to become preorganized into a more regular helical conformation. This will therefore favor complexation by lowering the entropic cost. Preferred strategies for increasing stacking include but are not limited to the addition of simple

substituents to DNA bases (e.g., methylation of pyrimidines at C-5), an increase of the surface area of DNA bases (e.g., by addition of aromatic heterocyclic groups to the C-5 position of pyrimidines), and the use of nonpolar DNA base analogues.

Preferred strategies for limiting bond rotations prior to complexation employ covalent bonds and include the synthesis of i) backbones with restricted freedom, ii) bicyclo-DNA, iii) hexose-DNA, and iiii) circular DNA. Prefered examples of nucleic acid derivatives with rigid backbones include peptide nucleic acid (PNA) analogues containing amide bonds. Since an amide has restricted rotation about the carbonyl-nitrogen bond, PNAs are capable of forming strong duplexes with DNA at lowered ionic strength and very strong triplexes even at normal ionic strength. In the bicyclo-DNA approach, the normally flexible furanose ring is rigidified by addition of an ethylene bridge from C-3' to C-5', thereby forming a second five-membered ring to the natural structure. Since five-membered rings are considerably more flexible than six-membered rings, DNA analogues have been synthesized in which the furanose ring is expanded to a six-membered ring. In some cases, such oligonucleotide analogues hybridized more strongly to DNA than the natural furanose-based structures. Another preferred way to significantly limit the conformational freedom of a flexible oligonucleotide is to cyclize the chain. A circular oligonucleotide can bind a single-stranded target RNA or DNA by forming standard Watson-Crick bonds. However, such binding is limited because of the helical twist of DNA.

In a more preferred embodiment, the linking of binding domains is used as strategy for preorganization. One aspect of critical importance for this strategy is the design of the linking group or groups. For optimal preorganization and thus highest affinity and selectivity, a linker should be both rigid and orient the binding domains in the productive geometry. Noncovalent links between binding domains have the advantage of simplifying the synthesis, but have the disadvantage of being relatively weak, thereby limiting effective preorganization. Preferred are covalent links between binding domains. For example, thiols may be placed into opposite strands of a duplex-forming sequence and used for disulfide cross-linking upon oxidation. Such duplexes become stabilized thermodynamically, presumably beause of the entropic benefit.

Using covalent links between domains, preorganized oligonucleotide structures can be prepared that lead to triplex formation on single-stranded targets. Triple-helical nucleic acid structures are known since 1957. A purine DNA base can form hydrogen-bonded contacts on two sides, one termed the Watson-Crick face and the

other Hoogsteen face. Thus, in duplex DNA, a purine stretch presents sites in the major groove for Hoogsteen complexation by a third strand. Single-stranded DNA can also serve as a target for triple helix formation, since a purine stretch can be bound on two sides by a molecule carrying both a Watson-Crick complementary domain and a Hoogsteen complementary domain. A second motif for triplex formation is the so-called purine motif, in which purine-purine-pyrimidine base triads are formed (Beal, P.A., and Dervan, P.B. Science 251, 1360, 1991). As with the previous motif, a purine strand is in the middle, with two other strands forming hydrogen-bonded contacts. In such a motif, the Watson-Crick complementary pyrimidine strand represents the target and the other two strands the binding domains which are preorganized by linking.

A simple way to link two such triplex-forming domains is to connect them with extra non-pairing nucleotides or by a non-nucleotide linker such as hexaethylene glycol (clamp or fold-back oligonucleotides). A preferred strategy is to link two triplex-forming domains by two loops at both ends using nucleotide loops or non-nucleotide linkers (circular and looped oligonucleotides). Clamp-like oligonucleotides have been shown to bind target sequences with an 11°C advantage in T_m, whereas closure of the clamp into a full circle gave a 19°C advantage.

The clamp and circular oligonucleotide approaches are strategies in which two DNA-binding domains are linked at their end or ends. Another preferred approach is to link them across the center, thereby generating molecules with an 'H'-form. Examination of the base triads involved in triple helix shows that a bridge can easily link two C-5 positions on pyrimidines in opposite strands. Experimental results have shown that such a molecule cross-linked by a disulfide bridge (via thiopropyne-substituted thymidine nucleosides) binds a target strand more strongly than a clamp-like oligonucleotide (Chaudhuri, N.C., and Kool, E.T. J. Am. Chem Soc. 117, 10434, 1995). In a more preferred embodiment, the same strategy is used to cross-link a circular oligonucleotide with a disulfide bridge across the center. Thermal denaturation studies have shown that such bicyclic oligonucleotides bind complementary DNA strands with extremely high affinity (see lit. cited above). Sequence selectivity was also found to benefit from this additional preorganization strategy.

In still another preferred embodiment, multiple binding domains are linked together (tethered DNA). For maximum cooperativity, a rigid linking domain is preferred. Although flexible linkers may not maximize affinity and selectivity, they may provide

utility in some cases. For example, flexible tethers may be used to link two DNA-binding sequences for hybridization to separate sites such as purine stretches separated by non-homopurine segments.

V.5. IMMOBILIZATION OF CAPTURE MOLECULES

The support serving for immobilization of capture may be the same support which carries the inactivated enzym, or it may be a different support. The support may have a flat structure, or it may have the form of beads or the like. It may be selected from containers, metallic, porous, impervious or fibrous matrices or membranes or the like, as is well-known in the art. Such matrices may be utilized as derivatized (e.g., by adsorption of polylysine, phenylalanine-lysine, or octadecylamine) or non-derivatized solid supports for immobilization of capture molecules. Specifically, if the detection system is an electrochemical detection system, the support may consist of gold microelectrodes spaced by insulators therebetween.

Immobilization of capture molecules or derivatives thereof may be accomplished non-covalently or covalently by any of the well-known chemical coupling methods. For most applications, covalent immobilization techniques are preferred. Electrically mediated coupling procedures are equally applicable for use with the current invention. Also applicable for this invention are non-covalent, non-adsorptive immobilization techniques. For example, capture molecules derivatized with biotin residues may be immobilized onto solid supports functionalized by adsorption or covalent binding of streptavidin beforehand.

V.5.1. Immobilization of proteinaceous capture molecules

Methods for immobilization of proteinaceous molecules have been for the most part based on simple adsorption, a phenomenon that most authors attribute to hydrophobic bonding. Simple adsorption techniques, however, have some limitations because the non-specific adsorption process affects the reproducibility. Some proteins are also so small that their adsorptive affinity is very low and others are conformationally altered by adsorption to the extent that they become nonfunctional. Furthermore, heavily glycosylated proteins often have low affinity for hydrophobic surfaces such as polystyrene. Therefore, for most applications covalent immobilization techniques are preferred.

In one embodiment, suitable cross-linking reagents such as carbodiimides, homo-, or heterobifunctional cross-linkers are used for covalent immobilization of

proteinaceous capture molecules to functionalized solid supports. For example, proteinaceous capture molecules may be coupled via their primary amine groups to amine-reactive solid supports (e.g., N-hydroxysuccinimide ester-derivatized solid supports). Alternatively, proteinaceous capture molecules may be first derivatized with amine-reactive heterobifunctional cross-linking reagents to introduce pyridyl disulfide groups (e.g., by reaction with N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)), maleimide residues (e.g., by reaction with N-succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB)), or iodoacetyl groups (e.g., by reaction with N-succinimidyl-6-[(iodoacetyl)amino]hexanoate (SIAX)). Such derivatized proteinaceous capture molecules may be coupled to sulfhydryl-derivatized solid supports. Vice versa, proteinaceous capture molecules containing a free sulfhydryl group may be coupled to solid supports derivatized with sulfhydryl-reactive residues such as pyridyl disulfide, maleimide, or iodoacetyl groups. Furthermore, proteinaceous capture molecules without a free sulfhydryl group may be first derivatized with amine-reactive heterobifunctional cross-linking reagents to introduce free or protected sulfhydryl residues such as thioester moieties (e.g., by reaction with succinimidyl acetyl-thiopropionate (SATP)). The thioester moieties can be deprotected by treatment with an excess of neutral hydroxylamine. Since the protecting acetyl groups can be removed without adding disulfide reducing agents like dithiothreitol, disulfides indigenous to native proteinaceous capture molecules will not be affected. This is an important consideration if disulfides are vital to the binding activity of proteinaceous capture molecules.

In another embodiment, non-covalent non-adsorptive immobilization procedures are applied. For example, biotinylated proteinaceous capture molecules may be immobilized onto solid supports functionalized by adsorption or covalent binding of (strept)avidin beforehand.

V.5.2. Immobilization of oligonucleotide capture molecules

The methods by which capture oligonucleotides may be derivatized with reactive residues for immobilization onto solid supports are numerous. Preferred methods include but are not limited to those which allow selective derivatization of the termini to guarantee efficient hybridization with target nucleic acids.

V.5.2.1. Chemical modification of the terminal 5'-phosphate group of capture oligonucleotides

In one embodiment, capture oligonucleotides containing a 5'-phosphate group are used which are derivatized with amine or sulfhydryl terminal spacer molecules for

immobilization onto amine-reactive or sulfhydryl-reactive solid supports (Hermanson, G.T. Bioconjugate techniques, Academic Press, San Diego, 1996). For example, the 5'-phosphate groups of capture oligonucleotides may be reacted with carbodiimide in the presence of imidazole to form active phosphorimidazolide intermediates. These derivatives are highly reactive with diamines or bis-hydrazide compounds, forming amine terminal spacer molecules via phosphoramidate linkages. Derivatization of the 5'-phosphate groups with cystamine creates an amine terminal spacer containing a disulfide group. Reduction of the cystamine-labeled oligonucleotide using a disulfide reducing agent releases 2-mercaptoethylamine and generates a terminal thiol group.

In another preferred embodiment, sulfhydryl groups are introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using S-triphenylmethyl O-methoxymorpholinophosphite derivatives of 2-mercaptoethanol, 3-mercaptopropan (1) ol, or 6-mercaptohexan (1) ol (Connolly, B.A., and Rider, P., Nucleic Acids Res. 13, 4485, 1985). After clevage from the resin and removal of the phosphate and base protecting groups, oligonucleotides are obtained which contain an S-triphenylmethyl group attached to the 5'-phosphate group via a two, three, or six carbon chain. The triphenylmethyl group can be readily removed with silver nitrate to give the free thiol. Alternatively, primary amino groups can be introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using N-monomethoxytrityl-O-methoxydiisopropylaminophosphinyl 3-aminopropan (1) ol (Connolly, B.A. Nucleic Acids Res. 15, 3131, 1987). After cleavage from the resin and removal of the phosphate and base protecting groups, a monomethoxytrityl-$NH(CH_2)_3PO_4$ - oligomer is obtained. The monomethoxytrityl group can be removed with acetic acid to give the amine-containing oligonucleotide.

V.5.2.2. Chemical attachment of nucleotide derivatives to the termini of capture oligonucleotides

In another preferred embodiment, nucleotide derivatives suitable for immobilization onto solid supports are incorporated into capture oligonucleotides during automated chemical oligonucleotide synthesis. For example, oligonucleotides may be derivatized at their 5'-terminus by coupling of a uridine moiety via a 5'-5' linkage using 2',3'-di-O-acetyluridine 5'-(2-cyanoethyl N,N-diisopropylphosphoramidite) (Kuijpers, W.H.A. et al., Bioconjugate Chem. 4, 94, 1993). After oxidation of the 2',3' cis-diol of the terminal uridine residue at the 5'-terminus by treatment with periodate, the derivatized oligonucleotide can be immobilized onto amine-containing solid supports via reductive amination. Other nucleotide derivatives such as N-6 or C-8

derivatives of dATP carrying protected amine or protected sulfhydryl groups are equally applicable for this invention.

V.5.2.3. Enzymatic attachment of nucleotide derivatives to the termini of capture oligonucleotides

In another embodiment, nucleotide derivatives containing reactive residues are incorporated into capture oligonucleotides by enzymatic means. Preferred examples of purine nucleotides include but are not limited to dATP derivatized with a reactive residue at its N-6 position or C-8 position via long linker arms. For example, 8-aminohexyl-dATP is a preferred derivative for coupling to the 3' terminal of DNA oligonucleotides by terminal transferase (Hermanson, G.T. (ed.) Bioconjugate techniques, Academic Press, San Diego, 1996). Preferred examples of pyrimidine nucleotides include but are not limited to dUTP and dCTP modified with a reactive residue at their C-5 position via long linker arms.

V.5.3. Immobilization of fold-back or looped preorganized capture oligonucleotides

Preorganized fold-back or looped capture oligonucleotides may be modified in the non-pairing nucleotide region with reactive groups by incorporation of nucleotide derivatives such as N-6 or C-8 derivatives of dATP carrying protected amine or protected sulfhydryl groups via long spacer arms. After deprotection, such derivatized preorganized fold-back or looped capture oligonucleotides can be immobilized onto amine- or sufhydryl-recative solid supports or further derivatized with heterobifunctional reagents. Alternatively, preorganized fold-back or looped capture oligonucleotides may be formed by non-nucleotide linker molecules providing a functional group for immobilization onto a solid support in addition to the other two functional groups required for the formation of a preorganized oligonucleotide structure by cross-linking of the termini. A convenient molecule from which to build trifunctional linker molecules is the amino acid L-lysine. Its three functional groups,α-carboxy, α-amino, and ε-amino, can be derivatized independently to contain three arms carrying different reactive groups.

V.5.4. Introduction of spacer molecules between solid support and capture oligonucleotides

In order to provide flexibility to oligonucleotides attached to solid supports, spacer molecules may be incorporated between the solid support and the oligonucleotide termini. Preferred spacer molecules are sufficiently long and flexible to allow efficient hybridization of the immobilized oligonucleotides with captured target nucleic acids.

Examples of suitable spacer molecules are derivatives of 6-aminohexanoic acid (6-[6-((amino)hexanoyl) amino] hexanoate), providing a spacer arm with 14 atoms, oligomeric derivatives of 6-aminohexanoic acid (6-[6-((amino)hexanoyl)amino]-hexanoate), and oligoethylene glycol derivatives (Levenson, C., and Chang, C. Nonisotopically labelled probes and primers. In: PCR Protocols: A Guide to Methods and Applications (M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White, eds.) pp. 99-112, Academic Press, San Diego, 1990). Utilization of oligoethylene glycol as spacer molecules offers several advantages since oligoethylene glycol provides water solubility and a great degree of freedom for the linked oligonucleotides. Preferred are heterobifunctional derivatives of oligoethylene glycol including but not limited to those providing an amine-reactive residue such as a succinimidyl ester on one end and a sulfhydryl-reactive residue such as a maleimide or vinylsulfone residue on the other end. Since vinylsulfone residues are hydrolytically stable, they represent especially useful reactive residues for a second coupling step. Useful are also derivatives of oligoethylene glycol containing a t-Boc-protected amine on one end and an unprotected amine or an amine-reactive residue on the other end. After reaction of the unprotected functional residue, the t-Boc protecting group can be easily removed by treatment with trifluoroacetic acid. Thus, a wide range of derivatives of oligoethylene glycol may be used as spacer molecules between the solid support and the oligonucleotide termini. Conjugation of capture oligonucleotides or derivatives thereof to spacer molecules may be accomplished by any of the well-known chemical coupling methods.

In one preferred embodiment, primary amino groups are introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using N-monomethoxytrityl-O-methoxydiisopropylaminophosphinyl 3-aminopropan (1) ol as mentioned above. Thereafter, the terminal amino group may be derivatized with an amine-reactive heterobifunctional reagent containing a long spacer arm such as succinimidyl 6-[3-(2-pyridyldithio) propionamido]hexanoate (LC-SPDP), succinimidyl 6-[6-{((iodoacetyl) amino) hexanoyl}amino]hexanoate (SIAXX), or oligoethylene glycol derivatives containing a succinimidyl ester on one end and a vinylsulfone residue on the other end. The sulfhydryl-reactive residue of such reagents may be used for coupling of the oligonucleotide-spacer conjugates to thiol-containing solid supports.

In another preferred embodiment, nucleotide derivatives containing reactive residues for covalent attachment of spacer molecules are incorporated into oligonucleotides by enzymatic means. For example, 8-aminohexyl-dATP may be utilized for coupling to the 3' terminal of DNA oligonucleotides by terminal transferase (Hermanson, G.T.

(ed.) Bioconjugate techniques, Academic Press, San Diego, 1996). The terminal amino group of the incorporated nucleotide may be further derivatized with heterobifunctional cross-linking reagents such as LC-SPDP, SIAXX, or oligoethylene glycol derivatives containing a succinimidyl ester on one end and a vinylsulfone residue on the other end. Using this approach, oligonucleotides can be derivatized at their 3'-terminus with a variety of spacer arms including 6-[6-((amino)hexanoyl)amino]-hexanoate and oligoethylene glycol derivatives of various length. The terminal sulfhydryl-reactive groups of such spacer molecules can be used for subsequent coupling of the oligonucleotide-spacer conjugates to sulfhydryl-containing solid supports. Alternatively, the pyridyl disulfide residues of LC-SPDP-derivatized oligonucleotides can be reduced to generate free sulfhydryl groups for subsequent coupling of the oligonucleotide-spacer conjugates to sulfhydryl-reactive solid supports.

In another preferred embodiment, oligonucleotides may be derivatized at their 5'-terminus by coupling of a uridine moiety via a 5'-5' linkage using 2',3'-di-O-acetyluridine 5'-(2-cyanoethyl N,N-diisopropylphosphoramidite) as mentioned above. After oxidation of the 2',3' cis-diol of the terminal uridine residue at the 5'-terminus by treatment with periodate, 6-[6-((amino)hexanoyl)amino]hexanoate or oligoethylene glycol derivatives containing an amine residue on one end and a carbyoxyl residue on the other end may be coupled to the aldehyde groups of the oligonucleotide via reductive amination. Thereafter, the terminal carboxyl residues of the spacer molecules may be activated for subsequent coupling of the oligonucleotide derivative to amine-containing solid supports.

In still another preferred embodiment, preorganized fold-back or looped oligonucleotides are modified in the non-pairing nucleotide region with reactive groups by incorporation of nucleotide derivatives such as N-6 or C-8 derivatives of dATP carrying protected amine groups via long spacer arms (e.g., a protected derivative of 8-aminohexyl-dATP). After deprotection, such derivatized preorganized fold-back or looped oligonucleotides may be further derivatized with heterobifunctional cross-linking reagents such as LC-SPDP, SIAXX, or oligoethylene glycol derivatives containing a succinimidyl ester on one end and a vinylsulfone residue on the other end. Alternatively, preorganized fold-back or looped oligonucleotides are formed by non-nucleotide linker molecules such as L-lysine providing a functional group for covalent attachment of spacer molecules in addition to the other two functional groups required for formation of the preorganized oligonucleotide structure. Preferred examples of trifunctional linker molecules

include but are not limited to L-lysine residues in which the α-amino and ε-amino groups are derivatized with hydroxyl terminal spacer molecules and the α-carboxyl group with derivatives of oligoethylene glycol spacer molecules containing a t-Boc-protected amine on one end and an unprotected amine on the other end. The terminal t-Boc protecting group can be removed by treatment with trifluoroacetic acid.

V.6. AFFINITY COMPONENTS

The liposomes to be used in the present invention contain encapsulated enzyme activators and are derivatized on the surface with affinity components capable of binding to analytes, analyte derivatives, or analyte-reactive molecules. Preferred affinity components include but are not limited to affinity components reactive with i) nucleic acids and derivatives thereof, ii) antigens and derivatives thereof, and iii) antibodies and derivatives thereof.

V.6.1. NUCLEIC ACID-REACTIVE AFFINITY COMPONENTS

In one embodiment of the present invention, the liposomes of the invention are derivatized on the surface with affinity components capable of binding to captured target nucleic acids or amplicons thereof. Preferred nucleic acid-reactive affinity components include but are not limited to single-stranded nucleic acids, single-stranded oligonucleotides, 'preorganized' oligonucleotide structures, intercalating agents, intercalating agents conjugated to single-stranded oligonucleotides or single-stranded nucleic acids, and immunoglobulins or fragments of immunoglobulins with specificity for double- and/or triple-stranded nucleic acids. The nucleic acids and oligonucleotides are designed for hybridization to single-stranded segments of captured target nucleic acids or amplicons thereof.

V.6.1.1. Intercalating agents

In one preferred embodiment of liposome-coupled affinity components, DNA intercalating agents are used. Intercalating agents are molecules which are characterized by planar aromatic ring structures of appropriate size and geometry that can be inserted between base pairs in double-stranded DNA. During intercalation neighboring base pairs in DNA are separated to allow for the insertion of the intercalating ring system, causing an elongation of the double helix by stretching. Examples include but are not limited to phenanthridines and acridines (e.g., ethidium bromide; propidium iodide; hexidium iodide; acridine orange; 9-amino-6-chloro-2-methoxyacridine (ACMA)), indoles and imidazoles (e.g., the bisbenzimide dyes Hoechst 33258 and Hoechst 33342; 4',6-diamidino-2-phenylindole (DAPI); 4',6-(diimidazolin-2-yl)-2-phenylindole (DIPI)), anthracyclines (e.g., doxorubicin;

daunorubicin), cyanine dyes (e.g., benzoxazolium-4-pyridinium dyes, benzothiazolium-4-pyridinium dyes; benzoxazolium-4-quinolinium dyes; benzothiazolium-4-quinolinium dyes), actinomycin D, hydroxystilbamidine, psoralens (e.g., 4 ,5',8-trimethylpsoralen (trioxsalen); psoralen; angelicine (isopsoralen)), and derivatives thereof. The various classes of intercalating agents are characterized by different binding affinity constants as well as by different preferences for base-pairs and DNA conformations.

It is preferred that the DNA intercalating agents used as affinity components possess preference for double-stranded and/or triple-stranded DNA. Such DNA intercalating agents include but are not limited to actinomycin D, anthracyclines, e.g. doxorubicin and daunorubicin, DAPI, and the bisbenzimide intercalators Hoechst 33258 and Hoechst 33342. DAPI associates in the minor groove of double-stranded DNA, preferentially binding to AT clusters, although there is evidence that DAPI also intercalates in those DNA sequences containing as few as two AT base pairs. Binding of DAPI to double-stranded DNA occurs with an approximately 20-fold fluorescent enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove. Fluorescence enhancement does not occur with single-stranded DNA. The relatively non-toxic and water-soluble (2%) bisbenzimide intercalators Hoechst 33258 and Hoechst 33342 are also minor groove-binding DNA intercalators with a preference to contiguous AT base pairs.

Altenatively, homodimers and heterodimers of intercalating agents may be used as affinity components. It has been shown that appropriately designed dimers of intercalating agents have DNA binding affinities several orders of magnitude greater than those of their parent compounds. For example, the intrinsic DNA binding affinity of ethidium bromide and ethidium homodimer are 1.5×10^5 M^{-1} and 2×10^8 M^{-1}, repectively. Recently, several sets of dimers of intercalating agents have been developed (Haugland, R.P. Handbook of fluorescent probes and research chemicals, M-T.Z. Spence, ed., Molecular Probes, Inc., Eugene, OR, USA, 1996). For example, in the dimeric cyanine dye TOTO-1 (Molecular Probes, Inc., Eugene, OR, USA) the positively charged side chains of two monomeric cyanine dyes TO-PRO-1 (Molecular Probes, Inc., Eugene, OR, USA) are covalently linked. TOTO-1 exhibits a higher affinity for double-stranded DNA than even the ethidium homodimers. NMR studies of TOTO-1 interactions with a double-stranded 8-mer indicated that it is a bis-intercalator with interactions in the minor groove and that it distorts the helix by unwinding it where the dye is bound. In addition, TOTO-1 exhibits strong sequence selectivity for the site CTAG.

In still another embodiment, intercalating agents covalently linked to oligonucleotides are used as affinity components. The oligonucleotides provide binding specificity by hybridizing to complementary single-stranded sequences of the target nucleic acids. In preferred assay procedures of this invention for detecting and quantifying target nucleic acids, capture oligonucleotides with a limited length are used. Thereby, only a part of the target nucleic acid is hybridized to the immobilized capture oligonucleotide, while the rest of the target nucleic acid remains in a single-stranded configuration. The intercalating agent should interact with the hybrid duplex structure formed by the nucleic acid target and the oligonucleotide-intercalating agent conjugate, provided the length of the linker is sufficient to allow for appropriate folding. This approach has been demonstrated with an acridine derivative (2-methoxy-6-chloro-9-aminoacridine) covalently linked to the 3'-phosphate of a series of oligodeoxythymidylates via a polymethylene linker [$(Tp)_n(CH_2)_mACr$] (Héléne, C. et al., Biochem. Soc. Transact. 14, 201, 1986). NMR analyses revealed that the acridine ring was intercalated between AT base pairs. Additional stabilization was achieved when the 3'-phosphate group was twice substituted by the acridine derivative and a positively charged group to form a phosphotriester (Asseline, U. et al., J. Biol. Chem. 260, 8936, 1985). Thus, preferred examples of oligonucleotide-intercalator conjugates include but are not limited to conjugates in which intercalating agents with a preference for double-stranded and/or triple-stranded DNA are covalently linked to oligonucleotides via flexible oligomethylene linkers. In a more preferred embodiment, oligonucleotides containing a 3'-phosphate group and a uridine residue at the 5'-terminus via a 5'-5'-linkage (prepared by automated oligonucleotide synthesis as described by Kuijpers, W.H.A. et al. above) are used. The 2',3' cis-diol of the terminal uridine residue at the 5'-terminus is oxidized by treatment with periodate and the resulting aldehyde functions are used to attach long spacer molecules via reductive amination for covalent coupling of the oligonucleotide derivatives to the surface of affinity liposomes. The 3'-phosphate group is derivatized with an intercalating agent (with a preference for double-stranded and/or triple-stranded DNA) using a flexible oligomethylene linker.

V.6.1.1.1. Actinomycin D and derivatives

As mentioned above, derivatives of actinomycin D may be used as intercalating agents. Actinomycin D has a phenoxazin-2-amino-3-one chromophore and two cyclic pentapeptide lactones attached to the 1,9-positions of the chromophore. It binds to dG-dC base pairs in double-standed DNA by intercalation of its chromophore and by hydrogen bonding and hydrophobic interactions of its peptide lactones. The

unsubstituted 3-oxo, and 4- and 6-methyl groups of the chromophore as well as the integrity of the cyclic pentapeptide lactones are necessary for the biochemical properties of actinomycin D. However, systematic studies of structural modifications have demonstrated that the molecule of actinomycin D can accomodate a number of well-defined modifications at the C-7- and N^2-sites while retaining its biochemical properties (Sengupta, S.K. et al., Med. Chem. 24, 1052, 1981). Even after substitution at the C-7-position with bulky groups via rotationally flexible linkers, the DNA binding properties of actinomycin D are retained. For example, actinomycin D and the actinomycin D analogue 7-[(3,4-dichlorobenzyl)amino]-actinomycin D intercalate into calf thymus DNA with apparent binding constants of $2.3 \times 10^7\ M^{-1}$ and $2.6 \times 10^7\ M^{-1}$, respectively. Apparently, the flexible NH-CH_2 linkage of the actinomycin D analogue allows for the formation and aids the stabilization of the intercalated complex with DNA. Preferred examples of actinomycin D derivatives for the preparation of affinity liposomes include but are not limited to actinomycin D analogues containing a reactive residue attached to the N^2- or C-7-position via rotationally flexible linker molecules.

V.6.1.1.2. Anthracyclines and derivatives

Further, derivatives of the anthracyclines doxorubicin and daunorubicin may be used as intercalating agents for the preparation of affinity liposomes. Doxorubicin is a glycoside antibiotic that differs from daunorubicin by a single hydroxyl group on C-14. Both molecules contain an aminosugar, daunosamine, linked through a glycosidic bond to the naphthacenequinone nucleus. They intercalate into the DNA double helix in such a fashion that the aglycone moiety is between the adjacent base pairs and parallel to them. The daunosamine moiety lies in the major groove of the double helix and the protonated amine of the aminosugar binds electrostatically to the negatively charged phosphate groups of the DNA. Both antracyclines intercalate into the DNA double helix with a binding affinity constant of approximately $10^6\ M^{-1}$, which is the same order of magnitude as those found for actinomycin and the acridines.

It is known that doxorubicin as well as daunorubicin derivatives containing a daunosamine moiety with a modified or substituted amino group are still able to form complexes with DNA. For example, the N-acetyl derivative of daunorubicin binds to DNA, athough less strongly than non-derivatized daunorubicin. The 3'-deamino-3'-(3-cyanomorpholinyl)-derivative of doxorubicin represents another example. In dilution experiments, the complex of this compound with DNA exhibits a typical irreversible binding (Menozzi, M., Vannini, E., Valentini, L., Penco, S., and

Arcamone, F. Stud. Biophys. 104, 113, 1984). Furthermore, the antracyclines cinerubin A and B containing pyrromycinone as aglycone and a trisaccharide instead of a monosaccharide are also capable of intercalating into DNA. Despite the presence of the bulky trisaccharide moiety, the stiffening and elongating effects of cinerubins on DNA resemble the changes produced by acridine dyes. Thus, examples of doxorubicin and daunorubicin derivatives for the preparation of affinity liposomes are derivatives containing a flexible spacer attached to the daunosamine moiety. In a preferred embodiment, the amine group of the daunosamine moiety is derivatized with 2-iminothiolane (Jue, R. et al., Biochemistry 17, 5399, 1978). The cyclic imidoester reacts with amines to form a stable, positively charged linkage and leaves the sulfhydryl group available for further coupling. Using this heterobifunctional reagent, the positive charge of the original amine is preserved and can bind electrostatically to the negatively charged phosphate groups of the DNA.

V.6.1.2. Oligonucleotides and oligonucleotide derivatives

In another preferred embodiment, oligonucleotides and oligonucleotide derivatives are employed as affinity components. The oligonucleotides are designed to form specific helical complexes with those regions of captured target nucleic acids that are still in a single-stranded configuration. In a more preferred embodiment, 'preorganized' oligonucleotide structures are used as affinity components. The rational for using such structures has been illustrated in detail above (in connection with the description of capture molecules).

In order to provide flexibility to oligonucleotides attached to the surface of affinity liposomes, spacer molecules will usually be incorporated between the liposomal surface and the oligonucleotide termini. Preferred spacer molecules are sufficiently long and flexible to allow efficient hybridization of the tethered oligonucleotides with captured target nucleic acids or amplicons thereof. Preferred examples of spacer molecules include those which have been described above in connection with the introduction of spacer molecules between solid support and capture oligonucleotides.

Derivatization of the oligonucleotides or derivatives thereof with spacer molecules may be accomplished by any of the well-known chemical coupling methods. Preferred methods include those which allow selective derivatization of the termini to guarantee efficient hybridization with target nucleic acids. In one preferred embodiment of oligonucleotide derivatization with spacer molecules, oligonucleotides containing a 5'-phosphate group are derivatized in a carbodiimide-mediated reaction

with derivatives of oligoethylene glycol spacer molecules containing a t-Boc-protected amine on one end and an unprotected amine on the other end. First, the 5'-phosphate groups are reacted with carbodiimide in the presence of imidazole to form active phosphorimidazolide intermediates. (Hermanson, G.T. Bioconjugate techniques, Academic Press, San Diego, 1996). These derivatives are highly reactive towards amine nucleophiles. Thereafter, the terminal t-Boc protecting group is removed by treatment with trifluoroacetic acid for subsequent coupling of the amine terminal oligonucleotide-spacer conjugates to amine-reactive lipid derivatives in the liposomal bilayer.

In another preferred embodiment, primary amino groups are introduced at the 5'-termini of oligonucleotides by automated solid-phase synthesis using N-monomethoxytrityl-O-methoxydiisopropylaminophosphinyl-3-aminopropan (1)-ol (Connolly, B.A. Nucleic Acids Res. 15, 3131, 1987). After cleavage from the resin and removal of the phosphate and base protecting groups, a monomethoxytrityl-$NH(CH_2)_3PO_4$ - oligomer is obtained. The monomethoxytrityl group can be removed with acetic acid to give the amine-containing oligonucleotide. Thereafter, the terminal amino group may be derivatized with an amine-reactive hetetobifunctional reagent containg a long spacer arm such as (LC-SPDP) or (SIAXX) for coupling to thiol-containing lipid components in the liposomal bilayer.

In another preferred embodiment, nucleotide derivatives containing reactive residues for covalent attachment of spacer molecules are incorporated into oligonucleotides by enzymatic means. Preferred examples of modified nucleotides include those derivatives in which the reactive residue is incorporated in a way that does not affect enzyme recognition and activity. Preferred examples of the purine nucleotides have been detailed above under "Enzymatic attachment of nucleotide derivatives to the termini of capture oligonucleotides". Preferred examples of the pyrimidine nucleotides include but are not limited to dUTP and dCTP modified with a reactive residue at their C-5 position via long linker arms. In a more preferred embodiment, 8-aminohexyl-dATP is utilized for coupling to the 3' terminal of DNA oligonucleotides by terminal transferase (Hermanson, G.T. (ed.) Bioconjugate techniques, Academic Press, San Diego, 1996). The terminal amino group may further be derivatized with LC-SPDP or SIAXX. Using this approach, the oligonucleotides are derivatized at their 3'-terminus with spacer arms which are similar in length as 6-[6-((amino)hexanoyl)amino]-hexanoate. The terminal pyridyl disulfide groups can be used for subsequent coupling of the oligonucleotide derivative to sulfhydryl-containing lipid molecules in the liposomal bilayer. Alternatively, the pyridyl disulfide

residues of LC-SPDP-derivatized oligonucleotides can be reduced to generate free sulfhydryl groups for subsequent coupling of the oligonucleotide derivative to sulfhydryl-reactive lipid molecules in the liposomal bilayer (e.g., pyridyl disulfide-, maleimide- or iodoacetyl-containing derivatives of phosphatidyl ethanolamine).

In another preferred embodiment, nucleotide derivatives suitable for covalent attachment of spacer molecules are incorporated into oligonucleotides during automated chemical oligonucleotide synthesis. For example, oligonucleotides may be derivatized at their 5'-terminus by coupling of a uridine moiety via a 5'-5' linkage using 2',3'-di-O-acetyluridine 5'-(2-cyanoethyl N,N-diisopropylphosphoramidite) as detailed above for chemical attachment of nucleotide derivatives to the termini of capture oligonucleotides. After the periodate treatment of the terminal uridine residue, 6-[6-((amino)hexanoyl)amino] hexanoate is coupled to the aldehyde groups of the oligonucleotide via reductive amination. Thereafter, the terminal carboxyl residues of the spacer molecules are activated with N-hydroxysuccinimide in a carbodiimide-mediated reaction for subsequent coupling of the oligonucleotide derivative to amine-containing lipid molecules (e.g., phosphatidyl ethanolamine) in the liposomal bilayer.

In still another preferred embodiment, preorganized fold-back or looped oligonucleotides are modified in the non-pairing nucleotide region with reactive groups during automated chemical oligonucleotide synthesis by incorporation of nucleotide derivatives such as N-6 or C-8 derivatives of dATP carrying protected amine groups via long spacer arms (e.g., a protected derivative of 8-aminohexyl-dATP). After deprotection, such derivatized preorganized fold-back or looped oligonucleotides are further derivatized with the heterobifunctional cross-linking reagent LC-SPDP. The terminal pyridyl disulfide groups can be used for subsequent coupling of the oligonucleotide derivative to sulfhydryl-containing lipid molecules in the liposomal bilayer. The pyridyl disulfide residues can also be reduced to generate free sulfhydryl groups for subsequent coupling of the oligonucleotide derivative to sulfhydryl-reactive lipid molecules in the liposomal bilayer (e.g., pyridyl disulfide-, maleimide- or iodoacetyl-containing derivatives of phosphatidyl ethanolamine). Alternatively, preorganized fold-back or looped oligonucleotides are formed by non-nucleotide linker molecules providing a functional group for covalent attachment of spacer molecules in addition to the other two functional groups required for formation of the preorganized oligonucleotide structure. A preferred molecule for the construction of such trifunctional linker molecules is the amino acid L-lysine. Its three functional groups,α-carboxy, α-amino, and ε-amino, can be derivatized

independently to contain three spacer arms wth different terminal reactive groups. Preferred examples of trifunctional linker molecules include L-lysine residues in which the α-amino and ε-amino are derivatized with hydroxyl terminal spacer molecules and the α–carboxyl group with amine terminal spacer molecules in a carbodiimide-mediated reaction.

V.6.1.3. Antibodies with specificity for double-stranded and/or triple-stranded nucleic acids

In another preferred embodiment, antibodies with specificity for double and/or triple helical structures of DNA are employed as liposome-attached affinity components. The term "antibody" refers to immunoglobulins of any isotype or subclass as well as any fragment (e.g., Fab' of Fv fragments) of the aforementioned. Antibodies of any source are applicable including polyclonal materials obtained from any animal species, monoclonal antibodies from any hybridoma source, and all immunoglobulins (or fragments) generated with the aid of viral, prokaryotic or eukaryotic expression systems. Biologic recognition molecules with specificity for double and/or triple helical structures of DNA other than antibodies are equally applicable for use with the current invention.

V.6.2. AFFINITY COMPONENTS REACTIVE WITH ANTIBODIES AND ANTIGENS

In another embodiment of the present invention, the affinity liposomes containing encapsulated enzyme activators are applied for detecting and quantifying antigens and antibodies such as microbial antigens and anti-microbial antibodies. In preferred assay procedures, high molecular weight antigens (e.g., proteins) to be analyzed are captured by binding to immobilized antibodies and detected in a sandwich-type assay procedure by affinity liposomes carrying surface-attached antibodies with specificity for the captured antigen. Similarly, antibodies to be analyzed are captured by binding to immobilized antigen molecules and detected by affinity liposomes carrying surface-attached antibodies with specificity for the captured antibody subclass.

In an alternative assay procedure, affinity liposomes containing encapsulated enzyme activators may be applied for detecting and quantifying low molecular weight antigens (haptens) such as hormones (e.g., polypeptides, steroids), therapeutic drugs and toxicologic compounds. Preferred assay procedures for detecting and quantifying haptens include the use of affinity liposomes carrying surface-attached hapten molecules. Such affinity liposomes are added in selectively limited quantities

to samples to be analyzed and compete with hapten molecules in the sample for the binding sites of hapten specific antibodies immobilized onto a solid support. Alternatively, hapten molecules are labeled with affinity components capable of binding affinity liposomes. Using this assay procedure, the labeled hapten molecules are added in selectively limited quantities to samples to be analyzed.

In order to provide flexibility to surface-attached antibody molecules and to guarantee accessibility of surface-attached antigen molecules (including hapten molecules) for specific antibody-antigen interactions, spacer molecules are incorporated between the liposomal surface and antibodies or antigen molecules. Preferred examples of sufficiently long and flexible spacer molecules have been described above; e.g. in connection with the binding of oligonucleotides to the liposomes.

The methods by which proteinaceous affinity components such as antibodies as well as low molecular weight compounds such as polypeptides and steroids may be derivatized and covalently coupled to the surface of liposomes are numerous and well known in the art. For example, affinity components containing nucleophilic moieties such as a primary amine, a thiol, or a hydroxyl group may be reacted with residues on the surface of liposomes that contain electrophilic moieties or have been derivatized with such a moiety. Examples of electrophilic moieties include but are not limited to alkyl halides, alkyl sulfonates, active esters such as N-hydroxysuccimide esters, aldehydes, ketones, isothiocyano, maleimido, and carboxylic acid chloride residues. Vice versa, residues on the surface of liposomes containing a nucleophilic moiety can be reacted with an electrophilic moiety on the affinity component. Thus, any of a wide range of functional groups on both the affinity components and residues on the surface of liposomes may be utilized for conjugation, provided these groups are complementary. Alternatively, conjugation may be performed using hetero- or homobifunctional cross-linking reagents. Suitable reactions would be well known to one skilled in the art based on the nature of the reactive groups that are available or have been introduced to the molecules and information about the binding site requirements of the affinity components.

V.6.3. AFFINITY SYSTEMS FOR BINDING OF AFFINITY LIPOSOMES TO ANALYTE-REACTIVE REAGENTS

In still another embodiment of the present invention, the affinity liposomes containing encapsulated enzyme activators are applied for detecting and quantifying specifically bound analyte-reactive reagents such as secondary antibodies derivatized with

affinity components which are capable of binding to complementary affinity components attached to the surface of affinity liposomes. Preferred affinity systems providing complementary affinity components include but are not limited to hapten / anti-hapten antibody systems, enzyme inhibitor / enzyme systems, and the biotin / (strept)avidin affinity system.

In preferred assay procedures utilizing analyte-reactive reagents for the detection of high molecular weight antigens, captured antigen molecules are detected with antigen-specific antibodies derivatized with affinity components which are capable of binding to complementary affinity components attached to the surface of affinity liposomes. Similarly, antibodies to be analyzed in assays utilizing analyte-reactive reagents are captured by binding to immobilized antigen molecules and detected by conjugates consisting of antibodies with specificity for the captured antibody subclass and affinity components which are capable of binding to complementary affinity components attached to the surface of affinity liposomes. In assay procedures utilizing analyte-reactive reagents for the detection of nucleic acids, target nucleic acids may be hybridized to immobilized capture oligonucleotides and detected by affinity component-containing detector oligonucleotides via hybridization to single-stranded segments of captured target nucleic acids or amplicons thereof. Subsequently, affinity liposomes carrying complementary surface-attached affinity components are employed to detect hybridized detector oligonucleotides.

The methods by which proteinaceous affinity components such as antibodies, enzymes, and (strept)avidin as well as low molecular weight affinity components such as haptens, enzyme inhibitors, and biotin residues may be derivatized and covalently coupled to analyte-reactive reagents or to the surface of liposomes are identical with those described in previous sections of this specification.

V.6.3.1. Hapten / anti-hapten antibody affinity systems

In one preferred embodiment, haptens and corresponding anti-hapten antibodies are used as affinity components for binding of affinity liposomes to analyte-reactive reagents. Due to the high affinity of some anti-hapten antibodies, hapten-anti-hapten antibody systems provide sensitive detection reagents. For example, human chromosomes have been probed by hapten-modified cosmid probes with a sensitivity equal to that achieved with biotinylated cosmid probes. The term "antibody" refers to immunoglobulins of any isotype or subclass as well as any fragment (e.g., Fab' of Fv fragments) of the aforementioned. Antibodies of any source are applicable including polyclonal materials obtained from any animal

species, monoclonal antibodies from any hybridoma source, and all immunoglobulins (or fragments) generated with the aid of viral, prokaryotic or eukaryotic expression systems. Non-biologic binding molecules such as "molecular imprints" (synthetic polymers with pre-determined specificity for binding or complex formation) are also applicable to the invention. Preferred examples of haptens include but are not limited to digoxin, digoxigenin, dinitrophenol (DNP), trinitrophenol (TNP), biotin, fluorescein, tetramethyl-rhodamin, Texas Red, dansyl residues, lucifer yellow, and Cascade Blue fluorophores. For all of these haptens corresponding high affinity anti-hapten antibodies are commercially available. Especially the very high affinity of anti-fluorescein antibodies makes fluorescein an ideal hapten for various detection schemes. Fluorescein-anti-fluorescein immunoassays have displayed a similar sensitivity as biotin-streptavidin methods in combination with low nonspecific binding.

V.6.3.2. Enzyme inhibitor / enzyme affinity systems

In another preferred embodiment, enzyme inhibitors and corresponding enzymes are used as affinity components for binding of affinity liposomes to analyte-reactive reagents. Several considerations are important for the choice of enzyme inhibitors suitable for use in the present invention. High affinity binding of the inhibitor to the corresponding enzyme is the most important requirement. The overall binding constant (K_{off}/K_{on}) should be in the low nanomolar to picomolar range to guarantee tight binding. Methotrexate represents one example of such an inhibitor. Methotrexate binds to dihydrofolate reductase (DHFR) with an overall binding constant of 2.1×10^{-10} M.

One preferred approach of increasing the affinity of enzyme inhibitors is the construction of multisubstrate adduct inhibitors (Broom, A.D. J. Med. Chem. 32, 2, 1989). In principle, such inhibitors can be designed for any enzyme that binds two or more substrates simultaneously (cofactors are considered to be substrates in this context). For example, multisubstrate adduct inhibitors for enzymes catalyzing bimolecular reactions can be synthesized by covalent conjugation of both substrates. Typically, the binding affinity of potent multisubstrate adduct inhibitors is 10^3-10^6 times the binding affinity of either substrate. Another preferred approach of increasing the affinity of inhibitor-enzyme interactions is to combine several covalently linked inhibitor molecules with an enzyme complex consisting of two or more copies of the enzyme. Provided the binding sites are in sufficiently close position, simultaneous binding of covalently linked inhibitor molecules is likely to occur leading to a significant increase of the apparent affinity of the inhibitor-enzyme interaction.

Further important considerations for the choice of suitable enzyme inhibitors include i) low molecular weight, ii) solubility in aqueous solutions, and iii) the ability of chemical conjugation of the inhibitor to analyte-reactive reagents or to the surface of liposomes without impairment of the binding affinity. Suitable for use in the invention are water-soluble, small molecular weight inhibitors. In one preferred embodiment, methotrexate (L-4-amino-N^{10}-methylpteroyl-glutamic acid), a water-soluble compound with a molecular weight of 508.5 daltons, is used as inhibitor of DHFR (dihydrofolate reductase). The γ-carboxyl group of the glutamate moiety of this inhibitor can be derivatized without impairing its binding affinity to the enzyme.

Although small molecular weight inhibitors are preferred, high molecular weight inhibitors are also included in this invention. For example, the placental ribonuclease inhibitor (RPI) is a 50 kD protein that forms tight complexes with ribonucleases. RPI inhibits RNase A with an extremely low K_i value of 4×10^{-14} M, approaching the affinity of avidin for biotin. Moreover, RPI binds to angiogenin, a blood vessel-inducing protein with 35% sequence homology to pancreatic RNase, with an even lower K_i value of 4×10^{-16} M.

Equally important considerations for the choice of suitable enzyme inhibitor / enzyme affinity systems include i) the molecular weight of the enzyme, ii) the accessibility of purified enzyme, and iii) the ability of chemical conjugation of the enzyme to analyte-reactive reagents or to the surface of liposomes without impairment of the inhibitor binding affinity. Preferred for use in the invention are enzymes with a low molecular weight which are abundantly present in a variety of organisms. DHFR represents one example of such enzymes. DHFR is found in microorganisms as well as in vertebrates and is easy to purify by affinty chromatography techniques using methotrexate-derivatized matrices. The molecular weight of DHFR of different sources varies between 18 kD and 24 kD. Mammalian DHFR are in general slightly larger (approx. 21 kD) than bacterial DHFR (approx. 18 kD). Furthermore, DHFR has been derivatized with heterobifunctional cross-linking reagents such as LC-SPDP (succinimidyl 6-[3-(2-pyridyldithio) propionamido]hexanoate) and conjugated to other proteins with good retention of methotrexate binding affinity.

V.6.3.3. Biotin-(strept)avidin affinity system

In still another preferred embodiment, biotin and (strept)avidin are used as affinity components for binding of affinity liposomes to analyte-reactive reagents. Avidin is a glycoprotein found in egg whites that contains four identical subunits of 16,400

daltons each, giving an intact molecular weight of approximately 66 kD. Each subunit contains one binding site for biotin. The biotin interaction with avidin is among the strongest non-covalent affinities known, exhibiting an affinity constant of about 1.3×10^{-15} M. The tetrameric native structure of avidin is resistant to denaturation under extreme chaotropic conditions. One disadvantage of the use of avidin, however, is its tendency to bind nonspecifically to components other than biotin due to its high pI of about 10 and carbohydrate content. The strong positive charge on the protein causes ionic interactions with more negatively charged molecules such as nucleic acids.

Streptavidin is another biotin binding protein from *Streptomyces avidinii* that can overcome some of the nonspecificities of avidin. Similar to avidin, streptavidin contains four subunits, each with a single biotin binding site. The intact tetrameric protein has a molecular mass of about 60 kD, slightly less than that of avidin. Like avidin, streptavidin is an extremely robust protein that can tolerate a wide range of buffer conditions, pH values, and chemical modification processes without loss of biotin binding activity. The primary structure of streptavidin, however, differs considerably from that of avidin despite the fact that they both bind biotin with similar avidity. This variation in the amino acid sequence results in a much lower pI of 5-6. Moderation in the overall charge of the protein substantially reduces the amount of nonspecific binding due to ionic interaction with other molecules. Of additional significance is the fact that streptavidin is not glycosylated. Thus, for the detection and quantification of nucleic acids streptavidin provides a significant advantage over avidin.

Biotin provides an additional advantage in that a large variety of biotin derivatives is commercially available which are suitable for covalent coupling to various reactive residues on analyte-reactive reagents and on the surface of liposomes.

V.7. AFFINITY LIPOSOMES

Liposomes are artificial structures primarily composed of phospholipid bilayers exhibiting amphiphilic properties. Other molecules, such as cholesterol, fatty acids, or lipid derivatives also may be included in the bilayer construction. The morphology of liposomes can be classified according to compartmentalization of aqueous regions between bilayer shells. If the aqueous regions are segregated by only one bilayer each, the liposomes are called unilamellar vesicles (ULV). If there is more than one bilayer surrounding each aqueous compartment, the liposomes are termed multilamellar vesicles (MLV). ULV are further classified according to their relative

size. Usually, the diameter of small unilamellar vesicles (SUV) is less than 100 nm with a minimum of about 25 nm, whereas the diameter of large unilamellar vesicles (LUV) is more than 100 nm with a maximum of about 2500 nm. MLVs typically form large complex honeycomb structures. As a consequence of the almost infinite number of ways each bilayer can be associated and interconnected with other bilayers, MLVs are difficult to categorize or exactly to reproduce. MLVs are the simplest to prepare, the most stable, and the easiest to scale up to large production levels. Although any kind of liposomes may be used in the present invention, the most useful form thereof consists of small, spherical ULVs containing hydrophilic enzyme activators that are protected from the outer environment by the lipid shell.

The outside surface of the liposomes of the present invention is derivatized to contain covalently attached molecules designed to target the liposomes for specific interactions with nucleic acids, antibodies, antigens, and derivatives thereof. Such liposomes are termed affinity liposomes in this invention.

V.7.1. Lipid components of affinity liposomes

Phospholipids are the most important constituents of affinity liposomes. Two main forms of lipid derivatives exist biologically, molecules containing a glycerol backbone and those containing a sphingosine backbone. Naturally occurring phospholipids can be isolated from a variety of sources including egg yolk. The composition of egg phospholipids, however, can vary considerably depending on age of the eggs, the diet of the chickens, and the method of processing. Furthermore, egg lecithin for example is not a single compound, but consists of a mixture of phosphatidyl cholines containing about 31% saturated fatty acid having a chain length of 16 carbons, 16% saturated fatty acid with 18 carbons, about 48% also with 18 carbons but having at least 1-2 points of unsaturation, and the rest a variety of other fatty acid constituents. Preferred for this invention are synthetic phospholipids of known chemical purity. Three major fatty acid derivatives of synthetic phospholipids are used primarily in affinity liposome preparation: (1) myristic acid (n-tetradecanoic acid; containing 14 carbons), (2) palmitic acid (n-hexadecanoic acid; containing 16 carbons), and (3) stearic acid (n-octadecanoic acid, containing 18 carbons).

Another significant component of affinity liposome preparations may be cholesterol. The presence of cholesterol in affinity liposome membranes has the effect of decreasing or even abolishing (at high cholesterol concentrations) the phase transition from gel state to the fluid or crystal state that occurs with increasing temperature. As a result, cholesterol modulates the permeability and fluidity of the

associated membrane, increasing both parameters at temperatures below the phase transition point and decreasing both above the phase transition temperature.

V.7.2. Lipid composition of affinity liposomes

One group of lipid compositions for the preparation of stable affinity liposomes of the present invention contains phosphatidyl ethanolamine (PE) derivatives. Examples include compositions with molar ratios of phosphatidyl choline (PC): cholesterol: negatively charged phospholipid (e.g., phosphatidyl glycerol, PG): derivatized PE of 8: 10: 1: 1. Another preferred composition using a maleimide derivative of PE without PG is PC: cholesterol: maleimide-PE of 85: 50: 15 (Friede, M., Van Regenmortel, M.H.V., and Schuber, F. Anal. Biochem. 211, 117, 1993). In a more preferred embodiment of this invention, the PE derivatives do not exceed a ratio of 1-10 mol PE per 100 mol of total lipid to maintain membrane stability.

One other group of liposomes may be used in the present invention which allows the release of encapsulated enzyme activators by a moderate increase of the ambient temperature. Liposomes are known to release encapsulated water-soluble contents more quickly near their liquid crystalline phase-transition temperature (T_m) than at other temperatures (Blok, M.C., van Deenan, L.L.M., and de Gier, J. Biochim. Biophys. Acta 433, 1, 1976). Such a temperature-sensitive release can be engineered by the selection of pure lipids that undergo sharp transition temperatures or by using mutually miscible mixtures of pure lipids to adjust the transition temperature to the desired point. Examples of such lipids are dipalmitoyl phosphatidyl choline (DPPC) (T_m = 41 °C), dipalmitoyl phosphatidyglycerol (DPPG) (T_m = 41 °C), and distearoyl phosphatidyl choline (DSPC) (T_m = 54 °C). The choice of lipids and the relative proportion of each depend upon the desired T_m and the size of the liposome. Small unilamellar vesicles (SUV) have apparent transition temperatures several degrees below those predicted from the T_m of the component lipids in large unilamellar vesicles (LUV) or in multilamellar vesicles (MLV). This effect is probably a result of stress in the highly curved bilayer structure in SUV. Typical lipid compositions of temperature-sensitive SUV, LUV, and MLV liposomes are described by various authors (for example, see Magin, R.L., and Weinstein, J.N. In: Liposome Technology, G. Gregoriadis, ed., vol. III., pp. 137-155, CRC Press, Boca Raton, Fl., 1984).

V.7.3. Methods for the preparation of affinity liposomes

Several methods are available to prepare affinity liposomes which are useful for this invention.One group of them comprises the following steps: (i) dissolving the lipid

mixture in organic solvent, (ii) dispersion in an aqueous phase containing enzyme activators, and (iii) fractionation to isolate the correct liposomal population. During all handling procedures the solutions are preferably protected from excessive exposure to light. Organic solvents are preferably maintained under a nitrogen or argon atmosphere to prevent introduction of oxygen. Water and buffers are preferably degassed using a vacuum and bubbled with inert gas before lipid components are introduced.

In the first step of these methods, the lipid components are dissolved in organic solvent (e.g., chloroform: methanol, by volume 2:1). This mixture will include any phospholipid derivatized with reactive groups or affinity components soluble in organic solvents as well as other lipids used to form the liposomal structure. Once the desired mixture of lipid components is dissolved and homogenized in organic solvent, one of several techniques may be used to disperse the liposomes in an aqueous solution containing enzyme activators. Preferred methods include (i) mechanical dispersion, (ii) detergent-assisted solubilization, and (iii) solvent-mediated dispersion.

Using mechanical dispersion methods for the preparation of affinity liposomes, an aqueous solution containing enzyme activators is added to the dried, homogenous lipid mixture and manipulated to effect dispersion. In a preferred embodiment, mechanical dispersion methods include but are not limited to simple shaking, non-shaken aqueous contact, high-pressure emulsification, sonication, extrusion through small-pore membranes, and various freeze-thaw techniques. Most of these methods result in a population of vesicles ranging from SUVs of only 25 nm diameter to very large MLVs.

Using detergent-assisted dispersion methods for the preparation of affinity liposomes, the amphipathic nature of detergent molecules is utilized to bring more effectively the lipid components into the aqueous phase for dispersion. The detergent molecules bind and mask the hydrophobic tails of lipids from the surrounding water molecules of the aqueous phase containing enzyme activators. Detergent treatment may be performed using a dried lipid mixture or small vesicles. In a preferred embodiment, non-ionic detergents such as the Triton X family, alkyl glycosides, or bile salts such as sodium deoxycholate are employed for this procedure. On removal of the detergent from the solution, the lipid micelles aggregate to form larger liposome structures. Liposomes of up to 100 nm containing a single bilayer may be created using detergent-asssisted methods.

Using solvent-mediated dispersion techniques for the preparation of affinity liposomes, the lipid mixture is first dissolved in a organic solvent to create a homogeneous solution and thereafter introduced into a aqueous phase containing enzyme activators. The solvent may or may not be soluble in the aqueous phase to effect this process. One preferred example of a solvent-mediated dispersion method is described by Batzri, S., and Korn, E.D. (Biochim. Biophys. Acta 298, 1015, 1973). Phospholipids and other lipids to be part of the liposomal membrane are first dissolved in ethanol. This ethanolic solution is then rapidly injected into an enzyme activator-containing aqueous solution of 0.16 M KCl using a Hamilton syringe, resulting in a maximum concentration of no more than 7.5% ethanol. Using this method, single bilayer liposomes of about 25 nm diameter can be formed. Other preferred solvent-mediated dispersion methods utilize solvents that are insoluble in the aqueous phase. The production of liposomes by this procedure involves the formation of a 'water-in-oil' emulsion. To create a proper reverse-phase emulsion, a small quantity of aqueous phase containing enzyme activators is introduced into a large quantity of organic phase containing the dissolved liposomes. The result is a milky dispersion. The emulsification process involves the use of mechanical means (shaking, stirring, or sonication) to effect the formation of small droplets of aqueous solution uniformly dispersed in the lipid-organic phase. Excess of organic solvent is then removed by rotary evaporation (reverse-phase-evaporation method) until the mixture becomes a viscous gel. To facilitate liquification of the gel, a small volume of aqueous phase is added. Finally, residual organic solvent and untrapped enzyme activators are removed by dialysis.

V.7.4. Encapsulation and release of enzyme activators from affinity liposomes

The encapsulation efficiency of water-soluble enzyme activators within affinity-liposomes depends on the liposome type. A comparative analysis of the encapsulation effiency in SUVs, LUVs, and MLVs of the water-soluble marker compounds 5(6)-carboxyfluorescein (CF) and tritiated cytosine-1-β-D-arabinofuranoside ([^{3}H]-Ara-C) has been performed by Magin, R.L. et al. in: Liposome Technology (G. Gregoriadis, ed.), vol. III., pp. 137-155, CRC Press, Boca Raton, Fl., 1984. Typical values of this study are given in Table III. For a fixed quantity of lipid, LUVs have the highest encapsulation efficiency.

Table III. Encapsulation characteristics of different types of liposomes

	LIPOSOME TYPE		
	SUV (DPPC:DSPC) (7:3)	LUV (DPPC:DPPG) (4:1)	MLV (DPPC:DPPG) (4:1)
Diameter (nm)	20 - 50	70 - 800	125 - 2000
Captured volume (l/mg lipid)	0.1 - 0.6	7 - 12	5 - 7
Encapsulation efficiency (%)	0.2 - 2.0	20 - 40	15 - 20

DPPC, dipalmitoyl phosphatidylcholine; DPPG, dipalmitoyl phosphatidyglycerol; DSPC, distearoyl phosphatidylcholine

In order to prepare enzyme activator containing liposomes, a suitable mixture of lipids is provided to obtain a stable liposomal layer which is preferably solved in an organic solvent. One method to prepare the said liposomes is to dry the lipid mixture, e.g. under reduced pressure at or close to ist transition temperature (the highest transition temperature of any one lipid in the mixture is taken into consideration) and to hydrate the lipid mixture with the enzyme activator dissolved in a suitable aqueous buffer. Vortexing or sonification assists the forming of liposomes. Another method to prepare the said liposomes is to provide the lipid mixture in a suitable organic solvent, to add the enzyme activator in an aqueous buffer, to sonicate the mixture until it appears homogeneous and to evaporate the organic phase, e.g. under reduced pressure (reverse-phase evaporation technique). The size of the liposomes thus obtained may be modified, e.g. SUV`s filled with enzyme activator may be converted into LUV`s. The liposomes may be separated from excess enzyme activator in their environment by a variety of methods, for example using gel chromatography or washing steps. Alternatively or in addition, they may be centrifuged or dialyzed against buffer. Further, the liposomes may be filtered, e.g. under pressure or with suction through a 0.3 to 1.0 µm pores containing filter. Via filtering, not only a separation from excess enzyme activator is possible, but also controlling their size and/or size distribution.

The release of encapsulated enzyme activators may be mediated by the addition of detergent (i.e., Triton X-100 or sodium deoxycholate) or organic solvent. Further, the release of encapsulated enzyme activators may be mediated by an increase of the ambient temperature (phase-transition release). A comparative analysis of phase-transition-mediated release of water-soluble marker compounds from different types of temperature-sensitive liposomes has been performed by Magin, R.L., and Weinstein, J.N. (In: Liposome Technology (G. Gregoriadis, ed.), vol. III., pp. 137-155, CRC Press, Boca Raton, Fl., 1984). Typical values obtained with the marker compounds 5(6)-carboxyfluorescein (CF) and tritiated cytosine-1-β-D-arabinofuranoside ([^{3}H]-Ara-C) are summarized in Table IV.

Table IV. Release characteristics of different types of temperature-sensitive liposomes

	LIPOSOME TYPE		
	SUV (DPPC:DSPC) (7:3)	LUV (DPPC:DPPG) (4:1)	MLV (DPPC:DPPG) (4:1)
Diameter (nm)	20 - 50	70 - 800	125 - 2000
Release (%) (1 min at 42°C)	5 - 10	30 - 60	30 - 60

DPPC, dipalmitoyl phosphatidylcholine (T_m = 41 °C); DPPG, dipalmitoyl phosphatidyglycerol (T_m = 41 °C); DSPC, distearoyl phosphatidylcholine (T_m = 54 °C)

Preferred heating rates at passage through T_m are 10 to 15 °C/min. Based on these data, detergent-mediated release of encapsulated enzyme activators may be the preferred release mechanism when ultimate detection sensitivity is required. The addition of detergent, however, requires to provide an additional container, whereas heating to approximately 45 °C is relatively easy to accomplish. Therefore, phase-transition-mediated release may be the preferred choice of release mechanism for less sensitive but for more cost-efficient assay procedures.

V.7.5. Covalent coupling of affinity components to liposomes

Different methods are available for covalent coupling of affinity components to liposomes. First, a purified lipid component (e.g., phosphatidyl ethanolamine or cholesterol) may be derivatized with an affinity component prior to incorporation into the lipid bilayer construction. Second, a purified lipid component may be activated prior to incorporation into the lipid bilayer construction and further derivatized with an affinity component after formation of the intact liposome. Third, the activation process and subsequent coupling step of an affinity component may be performed after formation of the intact liposome. Numerous cross-linking methods are available for derivatization of lipid functional groups. However, three main strategies are preferably used to couple affinity components to purified lipid components or lipid components incorporated into the lipid bilayer of liposomes: (i) heterobifunctional cross-linker-mediated conjugation reactions, (ii) carbodiimide-mediated reactions for coupling of amine residues to carboxyl groups or amine residues to phosphate groups, and (iii) coupling of amine residues to aldehydes via reductive amination.

V.7.5.1. Activation of lipid components with heterobifunctional reagents

The most common type of heterobifunctional reagents for the activation of lipid components includes amine- and sulfhydryl-reactive cross-linking reagents containing an N-hydroxy-succinimide (NHS) ester on one end and a maleimide, iodoacetyl, or pyridyl disulfide group on the other end. Examples of reagents used for activation of lipid components include those mentioned under item "immobilization of inactive enzyme molecules" and "immobilization of proteinaceous capture molecules". An additional example is m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). It is preferred to use cross linking reagents used to activate lipid components which contain long spacer molecules between the two functionalities. The length of the spacer is important in providing sufficient distance from the liposomal surface to ensure binding of the liposome-attached affinity component with another affinity partner. Preferred examples are LC-SPDP and SIAXX.

In one preferred embodiment, phosphatidyl ethanolamine (PE) is used as a component of the liposomes derivatized with heterobifunctional cross-linking reagents, preferably with those which derivatize the amino group of PE with a pyridyl disulfide group (e.g., by reaction with LC-SPDP), a maleimide residue (e.g., by reaction with SMPB, succinimidyl 4-(p-maleimidophenyl)butyrate), an iodoacetyl group (e.g., by reaction with SIAXX), a thioester moiety (e.g., by reaction with SATP, succinimidyl acetyl-thiopropionate), or an aldehyde function (e.g., by reaction with SFPA, succinimidyl-p-formylphenoxyacetate). Purified PE phospholipid may be

modified in organic solvent prior to incorporation into liposomes, or intact liposomes containing PE may be activated while suspended in an aqueous solution. It is more preferred that the PE derivative is prepared before the liposome is constructed. In this way, a stable stock preparation of modified PE can be made and used in a number of different liposome recipes. In a preferred embodiment, the uitilized PE is of a synthetic variety having fatty acid constitutents of dimyristoyl (DMPE), dipalmitoyl (DPPE), or distearoyl (DSPE) forms. For activation of pure PE, heterobifunctional reagents are preferred which are not modified with a sulfo residue at the N-hydroxysuccinimide ester moiety, since activation of PE is performed under non-aqueous conditions. If enzyme activators to be entrapped within the liposome are reactive with the PE derivatives, PE molecules are activated after formation of the liposomal structures to ensure derivatization of only the outer half of the lipid bilayer. In this case, heterobifunctional reagents are preferred which are modified with a sulfo residue at the N-hydroxysuccinimide ester moiety, since sulfo-derivatized cross-linking reagents cannot penetrate lipid bilayers.

V.7.5.2. Coupling of affinity components to activated lipid components

The methods by which derivatives of affinity components may be covalently coupled to cross-linker-derivatized lipid components are numerous and well known in the art. For example, affinity components containing nucleophilic moieties such as a primary amine or a thiol may be reacted with lipid components that have been derivatized with an electrophilic moiety. Examples of electrophilic moieties include but are not limited to alkyl halides, alkyl sulfonates, active esters such as N-hydroxysuccimide esters, aldehydes, ketones, isothiocyano, maleimido, pyridyl disulfide, and carboxylic acid chloride residues. Vice versa, lipid components or lipid derivatives containing a nucleophilic moiety can be reacted with an electrophilic moiety on the affinity component. Thus, any of a wide range of functional groups may be utilized for conjugation provided these groups are complementary.

V.7.5.3. Coupling of affinity components to intact liposomes

Derivatization of lipid components after formation of intact liposomes is the preferred method of this invention, if proteinaceous affinity components such as antibodies are covalently attached to the liposomal surface or enzyme activators to be entrapped within the liposomes are reactive with activated lipid components. In the latter case, lipid components such as PE molecules are activated after formation of the liposomal structures to ensure derivatization of only the outer half of the lipid bilayer. The methods by which affinity components may be covalently coupled to intact liposomes are similar to those employed for derivatization of lipid components and well known

in the art. The sulfo-N-succinimidyl ester (sulfo-NHS) variety of cross-linking reagents is preferred for activation of intact liposomes in aqueous suspension since they are incapable of penetrating membranes. Thus, only the outer surface of the liposomes will be modified.

Covalent attachment of proteinaceous affinity components (affinity proteins) such as antibodies may be performed with homobifunctional or heterobifunctional cross-linking reagents, with carbodiimides, by reductive amination, or by N-hydroxysuccinimide (NHS) ester activation of carboxylates. In one embodiment of the invention, affinity proteins are coupled via their primary amine groups to N-hydroxysuccinimide ester-derivatized palmitic acid incorporated into the bilayer construction as described by Huang, A. et al., (J. Biol. Chem. 255, 8015, 1980). In another embodiment, affinity proteins are coupled via their primary amine groups to DMS (dimethyl suberimidate)-derivatized PE molecules incorporated into the bilayer construction. DMS is a homobifunctional cross-linking agent containing amine-reactive imidoesters on both ends. The resulting amidine linkages are positively charged at neutral pH, thus maintaining the positive charge contribution of the original amine. In another preferred embodiment, affinity proteins are first derivatized with amine-reactive heterobifunctional cross-linking reagents to introduce pyridyl disulfide groups (e.g., by reaction with LC-SPDP), maleimide residues (e.g., by reaction with succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB)), or iodoacetyl groups (e.g., by reaction with SIAXX). Such derivatized affinity proteins are coupled to sulfhydryl-derivatized PE molecules incorporated into the bilayer construction. Vice versa, affinity proteins containing a free sulfhydryl group are coupled to incorporated PE molecules derivatized with sulfhydryl-reactive residues such as pyridyl disulfide, maleimide, or iodoacetyl groups. Affinity proteins without a free sulfhydryl group are first derivatized with amine-reactive heterobifunctional cross-linking reagents to introduce free or protected sulfhydryl residues such as thioester moieties (e.g., by reaction with succinimidyl acetyl-thiopropionate (SATP)). The thioester moieties can be deprotected by treatment with an excess of neutral hydroxylamine. Since the protecting acetyl groups can be removed without adding disulfide reducing agents like dithiothreitol, disulfides indigenous to the native affinity protein will not be affected. This is an important consideration if disulfides are vital to the binding activity of the affinity protein.

In still another embodiment of the invention, affinity proteins are used which are attached to the liposomal surface through the non-covalent use of high affinity interaction such as the biotin-(strept)avidin interaction. For example, biotinylated

liposomes may be complexed with biotinylated affinity proteins using (strept)avidin as bridging molecule or may be complexed with affinity proteins covalently conjugated with (strept)avidin. It is preferred to employ a long-chain spacer in constructing the biotin-PE derivative to allow sufficient spatial separation from the bilayer surface to accomodate (strept)avidin docking.

V.7.6. Storage of affinity liposomes

The most useful form of affinity liposomes for this invention consists of small, spherical ULVs containing surface-attached affinity components and entrapped hydrophilic enzyme activators. Small liposome vehicles, however, often aggregate on standing to form larger, more complex structures. Therefore, long-term storage of affinity-SUV in aqueous solution may not be possible without major transformations in liposome morphology. Freezing also fractures the liposomal membrane, releasing entrapped enzyme activators. In a preferred embodiment, cryoprotectants such as sugars or polyhydroxylic compounds are employed to prevent structural degradation problems on freezing.. The hydroxyl groups in cryoprotectants can take the place of water in hydrogen bonding activities, thus providing structural support even under conditions in which water is removed. Preferably, a procedure of Friede, M. et al., (Anal. Biochem. 211, 117, 1993) is used for long-term storage of affinity-SUVs. This procedure allows freezing and lyophilization of SUVs in the presence of 4% sorbitol with complete retention of liposome integrity on reconstitution.

V.8. SIGNAL AMPLIFICATION USING ADDITIONAL POLYMERIC CARRIER MOLECULES

The basic assay procedure of the present invention includes binding of the analyte to immobilized capture molecules by molecular biological or immunological interactions, detection of captured analytes by specific binding of affinity-liposomes containing encapsulated enzyme activators, removal of non-bound affinity-liposomes, release of enzyme activators encapsulated in specifically bound liposomes, activation of immobilized inactive enzymes and subsequent generation of optically active reporter molecules. The detection sensitivity achieved by this basic procedure will be sufficient for many applications since affinity liposomes with encapsulated enzyme activators allow excellent detectability of a binding event. However, for applications that require higher detection sensitivity, an amplification step may bepreferable.

In one specific embodiment ofthe present invention, additional polymeric carrier molecules are included in the assay procedure for signal amplification. Utilizing this

modified assay procedure, captured analytes are detected by hydrophilic polymers containing two different affinity components, one for specific binding to captured analytes and the other for binding of affinity liposomes. Since these polymers allow covalent attachment of multiple affinity components, each captured analyte molecule can bind multiple affinity liposomes leading to an effective signal amplification. Examples of affinity components useful in this embodiment have been mentioned above in connection with affinity componentes reactive with antibodies and antigens and for binding to analyte-reactive reagents.

Examples of synthetic and natural polymer derivatives include derivatives of polysaccharides, polyamino acids, polyvinyl alcohols, polyvinyl pyrrolidinones, polyacrylic acids, various polyurethanes, polyphosphazenes, and copolymers of these polymers. In a preferred embodiment, derivatives of dextran are employed as polymers.

The methods by which the above listed affinity components may be derivatized and covalently coupled to polymers are numerous and well known in the art. For example, affinity components containing nucleophilic moieties such as a primary amine, a thiol, or a hydroxyl group may be reacted with residues on polymeric carrier molecules that contain electrophilic moieties or have been derivatized with such a moiety. Examples of electrophilic moieties include, but are not limited to alkyl halides, alkyl sulfonates, active esters such as N-hydroxysuccimide esters, aldehydes, ketones, isothiocyano, maleimido, and carboxylic acid chloride residues. Vice versa, residues on polymeric carrier molecules containing a nucleophilic moiety can be reacted with an electrophilic moiety on the affinity component. Thus, any of a wide range of functional groups on both the affinity components and the polymeric carrier molecules may be utilized for conjugation provided these groups are complementary.

For covalent coupling of proteinaceous affinity components such as enzymes, antibodies, and streptavidin to polymeric carrier molecules, one strategy involves the use of hetero- or homobifunctional cross-linking reagents. For example, protein components may be derivatized with pyridyl disulfide groups (e.g., by reaction with SPDP) and subsequently coupled to sulfhydryl-containing polymeric carrier molecules via disulfide linkages. Alternatively, the introduced sulfhydryl residues may be reduced with disulfide reducing agents to create terminal sulfhydryl groups for coupling to sulfhydryl-reactive polymeric carrier molecules. If SPDP should affect the binding activity of one of these proteins, there are a number of additional cross-

linking reagents for coupling via disulfide bonds such as 2-iminothiolane (2-IT) and N-succinimidyl S-acetylthioacetate (SATA). 2-IT reacts with primary amines, instantly incorporating an unprotected sulfhydryl group. SATA also reacts with primary amines, but incorporates a protected sulfhydryl group, which is subsequently deacetylated using neutral hydroxylamine to produce a free sulfhydryl group. Other cross-linkers are available that can be used in different strategies for covalent coupling of proteinaceous affinity components to polymeric carrier molecules. S-(2-Thiopyridyl)-L-cysteine hydrazide (TPCH) and S-(2-thiopyridyl)mercaptopropiono hydrazide (TPMPH) react with the carbohydrate moieties of glycoproteins such as antibodies that have been previously oxidized by mild periodate treatment, thus forming a hydrazone bond between the hydrazide portion of the cross-linking reagent and the periodate-generated aldehydes. TPCH and TPMPH introduce pyridyl disulfide residues which can be used for coupling of the protein components to polymeric carrier molecules as described above. If disulfide bonding is unfavorable, other cross-linking reagents may be used. For example, N-(γ-maleimidobutyryloxy)succinimide (GMBS) and succinimidyl 4-(N-maleimidomethyl)cyclohexane (SMCC) react with primary amines, thereby introducing a maleimide group for coupling to sulfhydryl-containing polymeric carrier molecules via stable thioether linkages. Furthermore, cross-linking reagents may be used which introduce long spacer arms if steric hindrance problems interfere with the binding activity of the covalently coupled affinity component. Thus, there is an abundance of suitable cross-linking reagents which could be used. Suitable reactions would be well known to one skilled in the art based on the nature of the reactive groups that are available or have been introduced to the molecules and information about the binding site requirements of the affinity components.

The methods by which the above listed low molecular weight affinity components such as inhibitors, haptens, and biotin residues may be derivatized and covalently coupled to polymeric carrier molecules are also numerous and well known in the art. Several biotin and hapten derivatives are commercially available which provide hydrazide and sulfhydryl groups as well as amine- and sulfhydryl-reactive residues. Preferred examples of low molecular weight affinity components include but are not limited to D-biotinyl-ε-aminocaproic acid-N-hydroxysuccinimide ester and digoxigenin-3-O-methyl-carbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester for coupling to amine-containing polymeric carrier molecules and methotrexate-γ-hydrazide for coupling to aldehyde-containing polymeric carrier molecules.

As mentioned above, derivatives of dextran polymers are preferably employed as polymeric carrier molecules. Dextran is a naturaly occuring mainly linear polysaccharide consisting of repeating units of D-glucose linked together in glycosidic bonds wherein the carbon-1 of one monomer is attached to the hydroxyl group at the carbon-6 of the next residue. Occasional branch points also may be present in a dextran polymer, occurring as α-1,2, α-1,3, or α-1,4 glycosidic linkages. The monomers contain at least three hydroxyls that may undergo derivatization reactions. This multivalent nature of dextran allows affinity components to be attached at numerous sites along the polymer chain. Soluble dextran polymers with a molecular weight of 10 - 50 kD have been used in the past as modifying agents for proteins and other molecules including the application as a carrier of biotin residues and hapten molecules. Furthermore, dextran polymers have been used as a multifunctional linker to cross-link monoclonal antibodies with low molecular weight compounds.

In one preferred embodiment, dextran polymers are used which are oxidized with periodate to produce aldehydes. This procedures results in two aldehyde groups formed per glucose monomer, thus producing a highly reactive, multifunctional polymer able to couple numerous amine-containing molecules (Bernstein, A., Hurwitz, E., Maron, R., Arnon, R., Sela, M., and Wilchek, M. J. Natl. Cancer Inst. 60, 379, 1978). Polyaldehyde dextran may be conjugated with amine groups by Schiff base formation followed by reductive amination to create stable secondary (or tertiary amine) linkages. Amine-containing affinity components may be coupled to oxidized dextran polymers under mild conditions using sodium cyanoborohydride as reducing agent. The optimal pH for the reductive amination reaction is an alkaline environment between pH 7 and 10. In another preferred embodiment, dextran derivatives containing carboxy or amine-terminal groups are used for coupling of affinity components. Amine-terminal derivatives may be prepared by coupling diamine compounds such as ethylene diamine or diaminodipropyl amine (3,3"-imino*bis*propylamine) in excess to polyaldehyde dextran. Carboxyl-terminal derivatives may be prepared similarly by coupling molecules such as 6-aminocaproic acid or β-alanine to polyaldehyde dextran. Further, reactive alkyl halogen compounds containing a terminal carboxylate group on the other end such as chloroacetic acid or 6-bromohexanoic acid may be used. The carboxylates may then be aminated with ethylene diamine to form an amine-terminal spacer and further reacted with amine-reactive heterobifunctional reagents to prepare dextran derivatives carrying terminal sulfhydryl residues or sulfhydryl-reactive groups such as pyridyl disulfide, maleimide, or iodoacetyl groups. Such dextran derivatives are used

for covalent coupling of affinity components containing free sulfhydryl groups or derivatized with sulfhydryl-reactive residues.

V.9. SIGNAL AMPLIFICATION USING PREFORMED COMPLEXES OF AFFINITY LIPOSOMES

In another specific embodiment of the present invention, preformed complexes of affinity liposomes are utilized for signal amplification. Complexes of affinity liposomes may be prepared from affinity liposomes containing two types of surface-attached affinity components, one (type I) for specific binding to captured analytes and the other (type II) for complexation of affinity liposomes via bridging molecules providing at least two binding sites for type II affinity components. Examples of type I affinity components capable of binding to captured analytes have been enumerated above. Examples of type II affinity components for binding of affinity liposomes to bridging molecules have been detailed under "Affinity systems for binding of affinity liposomes to analyte-reactive reagents".

In a further specific embodiment of the present invention, complexes of affinity liposomes are used which are prepared from affinity liposomes containing two types of surface-attached affinity components, one (type I) for specific binding to captured analytes and the other (type II) for complexation of affinity liposomes via polymeric carrier molecules (e.g., derivatives of dextran polymers) containing covalently coupled affinity components which are capable of forming high affinity complexes with the surface-attached type II affinity components of affinity liposomes. The application of polymeric carrier molecules for complexation of affinity liposomes is specifically useful if affinity components containing only a single binding site for the corresponding affinity partner are utilized as bridging molecules. Examples of type I affinity components capable of binding to captured analytes have been enumerated above. Affinity components capable of forming high affinity complexes with affinity components covalently coupled to polymeric carrier molecules have been described under "affinity systems for binding of affinity liposomes to analyt-reactive reagents".

In still another embodiment of the invention, complexes of affinity liposomes are used which are prepared with two types of affinity liposomes containing different affinity components. The affinity components on type I affinity liposomes are capable of specifically binding to captured analytes (e.g., single-stranded oligonucleotides complementary to single-stranded segments of captured target nucleic acid, or antibodies with specificity for captured antigens). The affinity components on type II affinity liposomes are capable of specifically binding to the

affinity components of type I affinity liposomes (e.g., single-stranded oligonucleotides complementary to type I single-stranded oligonucleotides, or antibodies with specificity for the antibody subclass on type I affinity liposomes).

In specific amplified assay procedures of the invention utilizing preformed complexes of affinity liposomes, captured analytes (or specifically bound analyte-reactive reagents derivatized with affinity components) are detected by specific binding of preformed complexes of affinity liposomes containing surface-attached analyte-reactive affinity components (or affinity components capable of binding to affinity components conjugated to analyte-reactive reagents). Subsequent quantitation of enzyme activators encapsulated in specifically bound affinity liposome-complexes is performed as described.

In other specific amplified assay procedures, combinations of polymeric carrier systems and preformed complexes of affinity liposomes are employed for signal amplification. For example, captured target nucleic acids or amplicons thereof may be detected by dextran polymers containing two types of covalently linked affinity components, one being capable of specifically binding to a specific captured target nucleic acid or amplicons thereof in a structure restricted manner, and the other being capable of specifically binding preformed complexes of affinity liposomes. Subsequent quantitation of enzyme activators encapsulated in specifically bound complexes of affinity liposomes is performed as described.

V.9.1. Bridging molecules

Suitable bridging molecules include but are not limited to c) bi- or oligovalent anti-hapten antibodies or fragments thereof, as well as conjugates or fusion constructs thereof; b) enzyme conjugates and fusion constructs of enzymes providing more than one inhibitor-binding site; and a) avidin and streptavidin. Any bridging molecule that provides more than one binding site is useful for this type of amplification methodology.

V.9.1.1. (a) Use of (strept)avidin as bridging molecule

(Strept)avidin may be employed as bridging molecule for complexation of affinity liposomes carrying surface-attached biotin residues and low molecular weight analyte-reactive affinity components (e.g., low molecular weight nucleic acid-reactive components such as intercalating agents or oligonucleotides). Preferred complexes of affinity liposomes prepared via (strept)avidin include also complexes generated by reaction of (strept)avidin with affinity liposomes containing surface-attached

proteinaceous affinity components (e.g., antigen-specific antibodies for binding to captured antigens, high molecular weight antigens for binding to captured antibodies, antibodies with specificity for the subclass of captured antibodies, or antibodies with specificity for double- and/or triple-stranded nucleic acids) derivatized with biotin residues.

Streptavidin and avidin contain four biotin binding sites. Therefore, both molecules represent preferred multivalent bridging molecules for the synthesis of preformed complexes of affinity liposomes. Multivalent complexes formed by reaction of biotinylated liposomes with (strept)avidin have been shown to increase the detection sensitivity by one to two orders of.

V.9.1.2. (b) Use of antibodies as bridging molecules

In another preferred embodiment, high affinity anti-hapten antibodies or fragments thereof providing more than one hapten-binding site are used as bridging molecules for the synthesis of preformed complexes of affinity liposomes containing surface-attached hapten molecules and low molecular weight analyte-reactive affinity components (e.g., low molecular weight nucleic acid-reactive components such as intercalating agents or oligonucleotides). Preferred complexes of affinity liposomes prepared via oligovalent anti-hapten antibodies include also complexes generated by reaction of oligovalent anti-hapten antibodies with affinity liposomes containing surface-attached proteinaceous affinity components (e.g., antigen-specific antibodies for binding to captured antigens, high molecular weight antigens for binding to captured antibodies, antibodies with specificity for the subclass of captured antibodies, or antibodies with specificity for double- and/or triple-stranded nucleic acids) derivatized with hapten molecules.

In one preferred embodiment, anti-hapten IgG antibodies are utilized as bridging molecules. Intact IgG molecules provide two hapten binding sites and have been applied successfully for the preparation of soluble enzyme / anti-enzyme complexes such as peroxidase / anti-peroxidase (PAP) and alkaline phosphatase / anti-alkaline phosphatase (APAAP) complexes. In another preferred embodiment, anti-hapten antibodies of the IgM class providing 10 hapten binding sites per antibody molecule are employed. In another preferred embodiment, dimers or oligomers of IgG antibodies or hapten-binding fragments thereof are prepared by chemical conjugation methods using long heterobifunctional cross-linking reagents such as LC-SPDP (succinimidyl 6-[3-(2-pyridyldithio) propionamido]hexanoate). In still another preferred embodiment, two or more antibody binding regions with specificity

for the desired hapten are conjugated by genetic engineering technology and expressed as fusion proteins. Useful constructs of antibody binding regions include but are not limit to single chain fragments of variable antibody regions (scFv) containing on the same polypeptide chain V_H and V_L genes joined with a short linker DNA which codes for example for a flexible $(Gly_4Ser)_3$ peptide (Clackson, T. et al., Nature 352, 624, 1991). For dimeric anti-hapten scFv fusion proteins, the term 'fusion protein' refers to a genetically engineered protein whose coding region is comprised of the coding region residues of a first anti-hapten scFv molecule fused, in frame, to the coding region residues of a second anti-hapten scFv molecule. For the construction of oligomeric anti-hapten scFv fusion proteins, the dimeric anti-hapten scFv fusion protein construct is extended with coding region residues of additional anti-hapten scFv molecules as described for the dimeric fusion construct. Such constructs may also contain additional flexible oligopeptide linker fused, in frame, to the coding region residues of the scFv molecules.

V.9.1.3. (c) Use of enzymes as bridging molecules

In another preferred embodiment, enzyme conjugates providing at least two inhibitor binding sites are used as bridging molecule for the synthesis of preformed complexes of affinity liposomes containing surface-attached inhibitor molecules and low molecular weight analyte-reactive affinity components (e.g., low molecular weight nucleic acid-reactive components such as intercalating agents or oligonucleotides). Enzyme conjugates providing at least two inhibitor binding sites may also be used as bridging molecule for the synthesis of preformed complexes of affinity liposomes containing surface-attached proteinaceous affinity components (e.g., antigen-specific antibodies for binding to captured antigens, high molecular weight antigens for binding to captured antibodies, antibodies with specificity for the subclass of captured antibodies, or antibodies with specificity for double- and/or triple-stranded nucleic acids) derivatized with inhibitor molecules.

In a more preferred embodiment, dimers and higher oligomers of dihydrofolate reductase (DHFR) prepared by chemical conjugation methods are used as bridiging molecules. Preferred conjugation procedures use heterobifunctional cross-linking reagents containing long spacer molecules between the two functionalities such as succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate (LC-SPDP). In another preferred embodiment, two or more DHFR molecules, DHFR mutants, or methotrexate-binding DHFR fragments (all of these referred to as DHFR) are conjugated by genetic engineering technology and expressed as fusion proteins. DHFR from various sources has been cloned and expressed in prokaryotic

expression systems since the enzyme consists of a single polypeptide chain containing no disulfide linkages or posttranslational modifications (for example, see Prendergast, N.J. et al., Biochemistry 27, 3664, 1988). Due to these characteristics, DHFR represents a useful enzyme for the construction of fusion proteins. For dimeric DHFR fusion proteins, the term 'fusion protein' refers to a genetically engineered protein whose coding region is comprised of the coding region residues of a first DHFR molecule fused, in frame, to the coding region residues of a second DHFR molecule. For the construction of oligomeric DHFR fusion proteins, the dimeric DHFR fusion protein construct is extended with coding region residues of additional DHFR molecules as described for the dimeric fusion construct. Such constructs may also contain flexible oligopeptide linker fused, in frame, to the coding region residues.

V.9.2. Synthesis of affinity liposomes suitable for complexation

Affinity liposomes useful for the preparation of preformed complexes contain two types of surface-attached affinity components, one for specific binding to captured nucleic acids (or to polymeric carrier molecules) and the other for complexation of affinity liposomes (e.g. via bridging systems or via polymeric carrier molecules). The synthesis procedures of such affinity liposomes are identical with those described in previous sections of the present specification. Although two types of lipid derivatives are incorporated into the lipid bilayer construction, the overall molar ratios of derivatized lipid components versus non-derivatized lipid components remain unchanged in preferred lipid compositions. In one preferred embodiment, both affinity components are covalently attached to purified lipid components prior to incorporation into the same lipid bilayer construction. In another preferred embodiment, purified lipid components derivatized with different reactive residues are incorporated into the same lipid bilayer construction and then reacted with the two affinity components carrying different complementary functionalities. In still another preferred embodiment, the activation process and subsequent coupling step of the two affinity component is performed after formation of the intact liposome.

V.10. Performance of method

Important steps in performing the assay procedure of the present invention are; (i) contacting a sample which may contain the analyte to be detected with capture molecules linked to a solid support, (ii) contacting enzyme activator-containing affinity liposomes capable of binding to captured analyte (or to a structure formed between capture molecules and captured analyte, or a structure, e.g. a polymeric carrier molecule, bound to the captured analyte/capture molecules) with capture

molecules which had been brought into contact with the sample, (iii) separating unbound affinity liposomes (iv) providing release of enzyme activator from affinity liposomes bound to capture analyte into their environment, e.g. a solution, (v) bringing said environment into contact with immobilized inactive enzyme in order to reactivate the enzyme with enzyme activator released from the affinity liposomes in case they have been bound to captured analyte, (vi) contacting the immobilized enzyme with a substrate which is converted into reporter molecules in case the enzyme is activated, and (vii) measuring, optionally quantifying, the presence of the said reporter molecules.

As detailed previously in this specification, single steps of this method may be performed separately as desired. The sample may be diluted or not, dilution may be with water or buffer or a stabilized solution as required. The affinity liposomes may be added to the sample prior to its contact with the capture molecules or afterwards. Alternativley, after contacting the capture molecules with the analyte containing sample, washing steps may take place before the affinity liposomes are added. The time limit within which the affinity liposomes are to be added is not critical as long as the analyte is stably captured by the capture molecules. In some cases, the captured analyte may even be frozen and thawed again before further steps are performed. The affinity liposomes may be added per se or in a suitable solution or emulsion. Again, it might be possible to store the complex of affinity liposomes and capture molecules/analyte before the interior of the affinity liposomes is released. After separating non-bound affinity liposomes and release of the interior of bound affinity liposomes in a suitable environment, e.g. a buffered or optionally otherwise stabilized solution, this solution may be stored until it is used to activate immobilized inactive enzyme molecules. Care should be takent that this environment is free of metal ions or other substances which could result in a restoration of the enzyme activity independent of the presence of enzyme activator released from affinity liposomes. Restoration of activation of immobilized enzyme by enzyme activator released from affinity liposomes may be performed while reporter molecules are already present. Alternatively, the reporter molecules may be added to the already activated immobilized enzyme molecules. Measurement of substrate enzymatically converted from reporter molecules may be performed immediately after ist formation or later, if this is desired and the reporter molecules are sufficiently stable.

Since the shape of the supports which are covered with capture molecules or inactivated enzyme molecules and the presence of electochemical detecting means may be chosen in a broad variety as detailed above in this specification, it is clear

that the assay procedure of the present invention allows for a multiplicity of variations in regard to subsequency of steps, shape and number of vessels or plates or the like, and of time. This allows for adaption to any situation required for the procedure, e.g. for immediate measurement on one side, or for collecting a large number of samples to be analyzed during a longer time period even if the analyte itself is not stable in the sample on the other side, because a stable intermediate, e.g. released enzyme activator or substrate, may be safely stored before the last procedural steps are to be performed.

VI. SPECIFIC EXAMPLES

VI.1. PREPARATION OF INACTIVE ENZYMES

VI.1.1. Preparation of inactive metal-free enzymes

Removal of metal ions from metalloenzymes may be performed by treatment with chelating agents. Some metal complexing agents useful for this invention are listed in Table V.

Table V. Metal complexing agents employed to inhibit metalloenzymes (Auld, D.S. Meth. Enzymol. 158, 110, 1988)

Metal ion	Ligand	Log K_1	Log K_2	Log K_3	Log β_2	Log β_3
Zn^{2+}	1,10-Phenanthroline (phen)	6.6	5.8	5.2	12.4	17.6
	4,7-Dimethylphen	6.9	6.2	6.0	13.1	19.1
	α,α'-Bipyridyl	5.3	4.5	3.8	9.8	13.6
	EDTA Ethylenediamine tetraacetic acid	16.4				
	EGTA [(Ethylenedioxy)diethylene-dinitrolo] tetraacetic acid	12.9				
	Dipicolinic acid	6.4	5.5		11.9	
Cu^{2+}	1,10-Phenanthroline	9.3	6.8	5.4	16.1	21.5
	α,α'-Bipyridyl	8.2	5.5	3.3	13.7	17.0
	EDTA	18.8				
	EGTA	17.7				
Fe^{2+}	1,10-Phenanthroline					21.3
	α,α'-Bipyridyl	4.2	3.7	9.6	7.9	17.5
	EDTA	14.3				
	EGTA	11.9				
Fe^{3+}	EDTA	25.1				
	EGTA	20.5				

The apparent stability constant for di- and tridentate species are $\beta_2 = K_1K_2$, and $\beta_3 = K_1K_2K_3$

Utilizing chelating agents, the removal of metal ions from enzymes may occur by two mechanisms. One mechanism, generally a time-dependent process, involves depletion of the free metal ion concentration by the chelating agent leading to the dissociation of metal ions from the enzyme. The other mechanism, generally a rapid process, involves formation of a transient ternary complex of the chelating agent with

the metal and the enzyme with subsequent removal of the metal ion from the enzyme.

VI.1.1.1. Preparation of metal-free alkaline phosphatase

Zinc-containing alkaline phosphatase is prepared from *E. coli* (89 kD) as described by Lazdunski, C., and Lazdunski, M. (Biochim. Biophys. Acta 147, 280, 1967). The stock solution of the enzyme is stored in 20 mM Tris-HCl, pH 8.0, at -20 °C. The enzymatic activity is determined employing 4-nitrophenyl phosphate (1mM) in 10 mM Tris-HCl, 1.0 M NaCl, pH 8.0, as substrate.

The apoenzyme is prepared as described by Chappelet-Tordo, D., Iwatsubo, M., and Lazdunski, M. (Biochemistry 13, 3754, 1974). Zinc-containing alkaline phosphatase from *E. coli* is incubated at 25 °C overnight in 50 mM EDTA, pH 6.5, at a concentration of 1 mg/ml, and then passed through a Sephadex G25 column equilibrated with 50 mM EDTA, pH 6.5. Pooled fractions are concentrated to 30-40 mg/ml and passed through a Sephadex G25 column equilibrated with 10 mM N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES), pH 7.5. The zinc-free apoenzyme is completely inactive.

Alternatively, zinc-free apoenzyme may be prepared by treatment with Chelex 100 (200-400 mesh) in 1M Tris-HCl, pH 8.0, as described by Csopak, H. (Eur. J. Biochem. 7,186, 1969). The resin is equilibrated with the buffer (metal-free), then added to the native enzyme in metal-free buffer, so that the final volume is about 20% resin. The slurry is gently mixed until loss of enzymatic activity is virtually complete. Thereafter, the resin is removed by centrifugation at 10,000 x g. Using this procedure, the loss of alkaline phosphatase activity is time-dependent indicating that Chelex 100 is acting by depleting free metal ions from solution. The advantage of this method is that it allows the apoenyzme to be stored in the presence of the chelating agent, thus minimizing contamination by adventitious metal ions.

Active metallophosphatase is obtained by reconstitution on addition of a five-fold molar excess of $ZnSO_4$ to the apoenzyme in 10 mM TES, pH 7.8, at room temperature (Chappelet-Tordo, D., Iwatsubo, M., and Lazdunski, M. (Biochemistry 13, 3754, 1974).

VI.1.1.2. Preparation of metal-free aminopeptidase

Aeromonas aminopeptidase is an unusually stable monomeric enzyme of 29.5 kD that binds two equivalents of zinc ion. The enzyme can be obtained in large

quantities. The enzyme activity is determined using 37.5 μM L-alanine-*p*-nitroanilide in 50 mM HEPES, pH 7.5, as substrate (Auld, D.S. Meth. Enzymol. 158, 110, 1988).

The apoenzyme is prepared as described by Auld, D.S. (Meth. Enzymol. 158, 110, 1988). Zinc-containing aminopeptidase (0.5 mM) is dialyzed against three changes of 25 volumes of 2 mM 1,10-phenanthroline in 50 mM tricine, 50 mM KCl, pH 7.5. In the presence of 1,10-phenanthroline, the enzymatic activity of *Aeromonas* aminopeptidase is inhibited immediately. Apoaminopeptidase is freed from the chelating agent by dialysis against a minimum of 7 changes of 25 volumes of 50 mM HEPES, pH 7.5, over a 48 hr period. To confirm the complete removal of residual 1,10-phenanthroline, the absorption spectrum (200 - 400 nm) of the final dialyzing fluid is analyzed (1,10-phenanthroline: $\varepsilon_{265} = 3.15 \times 10^4\ M^{-1}\ cm^{-1}$).

Active metalloaminopeptidase is obtained by reconstitution on addition of a two-fold molar excess of $ZnSO_4$ to the apoenzyme (10^{-6} M) in 50 mM HEPES, pH 7.5, at room temperature (Auld, D.S. Meth. Enzymol. 158, 110, 1988).

VI.1.1.3. Preparation of metal-free carboxypeptidase A

Metal-free carboxypeptidase A is prepared as described by Auld, D.S. (Meth. Enzymol. 158, 110, 1988). An aqueous solution of the native enzyme (0.5 mM) is dialyzed against three changes of 25 volumes of 2 mM 1,10-phenanthroline in 50 mM tricine, 50 mM KCl, pH 7.5. Apocarboxypeptidase A is freed from the chelating agent by dialysis against a minimum of 7 changes of 25 volumes of 50 mM HEPES, pH 7.5, over a 48 hr period. To confirm the complete removal of residual 1,10-phenanthroline, the absorption spectrum (200 - 400 nm) of the final dialyzing fluid is analyzed (1,10-phenanthroline: $\varepsilon_{265} = 3.15 \times 10^4\ M^{-1}\ cm^{-1}$).

Alternatively, apocarboxypeptidase A is prepared by removing the metal ions from crystalline enzyme. A suspension of carboxypeptidase A crystals (5 mg/ml) is suspended in 10 mM 1,10-phenanthroline dissolved in 1 mM MES, pH 7.0, and incubated for 1 hr. The chelation treatment is repeated three times. Thereafter, the crystals are washed for 30 min with at least 4 changes of 1 mM metal-free MES, pH 7.0.

Active metallocarboxypeptidase is obtained by reconstitution on addition of a three-fold molar excess of $ZnSO_4$ to the apoenzyme (10^{-6} M) in 50 mM HEPES, pH 7.5, at room temperature (Auld, D.S. Meth. Enzymol. 158, 110, 1988). The enzyme activity

is determined using furylacryloylglycyl-L-phenylalanine in 50 mM HEPES, 1.0 M NaCl, pH 7.0, as substrate (Auld, D.S. Meth. Enzymol. 158, 110, 1988).

VI.1.2. Preparation and complementation of β-galactosidase mutant M15

The M15 β-galactosidase deletion mutant (α-acceptor) of *E. coli* has no enzyme activity, but binds as strongly as native β-galactosidase (the enzyme is tetrameric, being composed of four identical subunits of 135 kD, each with an active site) to a β-galactoside affinity column, and therefore must have a substrate binding site (for a review, see Ullmann, A., and Perrrin, D. In: The lactose operon (Zipser, D., and Beckwith, J.R., eds.) pp. 143-172, 1970). Addition of the 90-residue peptide CB2 (10.3 kD) containing residues 3 to 92 of β-galactosidase restores the activity of the M15 deletion mutant to at least two-thirds of the level of native β-galactosidase (Langley, K.E. et al., J. Biol. Chem. 250, 2587, 1975).

VI.1.2.1. Preparation of M15 extracts

M15 extracts are prepared from E. coli strain DZ 291(M15/F'M15) as described by Langley, K.E. (see VI.1.2.). Cells are broken by sonication and the suspension is centrifuged to remove debris. To the supernatant streptomycin sulfate is added to a final concentration of 5% (w/v) and after standing overnight at 4 °C, the supernatant is centrifuged and the pellet is discarded. To the supernatant ammonium sulfate is added to a final concentration of 50% and the M15-containing precipitate is dialyzed against 50 mM sodium phosphate, 5 mM β–mercaptoethanol, pH 7.2. The final protein concentration is approximately 30 mg/ml.

VI.1.2.2. α-Complementation

α-Complementation of the M15 deletion mutant with peptide CB2 is performed as described by Langley, K.E. (see. VI.1.2). Peptide CB2 is dissolved in 30% acetic acid, mixed with 100 μg of bovine serum albumin, and dried in vacuo. M15 extract (150 μg of protein in 50 mM sodium phosphate, 5 mM β-mercapto-ethanol, pH 7.2) and 100 mM sodium phosphate, 1.0 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0, are added to give a final volume of 200 μl, and the reaction mixture is incubated at room temperature. The enzyme activity is determined using o-nitrophenyl-β-D-galactopyranoside (2.5 mM) in 100 mM sodium phosphate, 1 mM $MgCl_2$, 100 mM β-mercaptoethanol, pH 7.5, as substrate (Craven, G.R., et al., J. Biol. Chem. 240, 2468, 1965).

VI.2. ENZYMATIC GENERATION OF OPTICALLY ACTIVE REPORTER MOLECULES

VI.2.1. Generation of reporter molecules by activated alkaline Phosphatase

VI.2.1.1. Generation of chromogenic reporter molecules

If alkaline phosphatase is the enzyme to be activated in the present invention, p-nitrophenyl phosphate (NPP) may be used as substrate which is converted to the yellow compound ("reporter molecule") p-nitrophenol which can be detected at 405 nm. The NPP substrate solution may suitably contain e.g. 3 mM NPP (Sigma-Aldrich, Deisenhofen, Germany) in 0.05 M Na_2CO_3. The substrate solution should be Zn^{2+}- and Co^{2+}-free and may be stored at 4°C. Using an increase of the ambient temperature for the release of liposome encapsulated $ZnCl_2$, the substrate solution may be added to immobilized enzyme molecules prior to the release of enzyme activator (e.g. as simultaneous washing step for removal of non-bound affinity liposomes). Using liposome-lysing solvents for the release of liposome encapsulated $ZnCl_2$, the substrate solution is preferably added to immobilized enzyme molecules together with the liposome-lysing solvent.

VI.2.1.2. Generation of fluorescent reporter molecules

Instead, activated alkaline phosphatase may be used to hydrolyze 4-methylumbelliferyl phosphate (MUP) into reporter molecules (4-methylumbelliferone) which are quantified using a 365 nm excitation filter and a 450 nm emission filter. The MUP substrate solution may suitably contain e.g. 0.2 mM MUP (Sigma-Aldrich, Deisenhofen, Germany) in 0.05 M Na_2CO_3. The substrate solution shoule be Zn^{2+}-and-Co^{2+} free and can be stored at room temperature. Using an increase of the ambient temperature for the release of liposome encapsulated $ZnCl_2$, the substrate solution is preferably added to immobilized enzyme molecules prior to the release of enzyme activator (e.g. as simultaneous washing step for removal of non-bound affinity liposomes). Using liposome-lysing solvents for the release of liposome encapsulated $ZnCl_2$, the substrate solution amy be added to immobilized enzyme molecules together with the liposome-lysing solvent.

VI.2.2. Generation of reporter molecules by activated β-galactosidase

VI.2.2.1. Generation of chromogenic reporter molecules

If M15 β-galactosidase is the enzyme to be activated in the present invention, 2-nitrophenyl-β-D-galactoside (NPG) is a useful substrate wich is converted to the

yellow compound p-nitrophenol, detectable at 405 nm. The NPG substrate solution may suitably contain 3 mM NPG (Sigma-Aldrich, Deisenhofen, Germany) in 100 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.5. The substrate solution may be stored at 4°C. Addition of the substrate solution may be as described under VI.2.1..

VI.2.2.2. Generation of fluorescent reporter molecules

Instead, activated M15 β-galactosidase molecules may be used to convert 4-methylumbelliferyl-β-D-galactopyranoside (4MUG). Generated reporter molecules (4-methylumbelliferone) are quantified using a 365 nm excitation filter and a 450 nm emission filter. The 4MUG substrate solution may suitably contain e.g. 0.2 mM 4MUG (Sigma-Aldrich, Deisenhofen, Germany) in 100 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.5. The substrate solution can be stored at room temperature. Addition of the substrate may be as described under VI.2.1.

VI.2.3. Generation of reporter molecules by activated phosphodiesterase

VI.2.3.1. Generation of chromogenic reporter molecules

If phosphodiesterase I (venom exonuclease) is the enzyme to be activated in the present invention, p-nitrophenyl thymidine-5'-phosphate (NPTP) or bis(p-nitrophenyl) phosphate (BNPP) may be used as substrate which are converted to the yellow compound p-nitrophenol, detectable at 405 nm. The substrate solution may suitably contain 3 mM NPTP (Sigma-Aldrich, Deisenhofen, Germany) or 3 mM BNPP (Sigma-Aldrich, Deisenhofen, Germany) in 100 mM Tris-HCl, 100 mM NaCl, pH 8.9. The substrate solution should be Mg^{2+}-free and may be stored at 4°C. Addition of the substrate may be as described under VI.2.1.

VI.3. ENZYMATIC GENERATION AND QUANTIFICATION OF REDOX MEDIATORS

Suitable substrates for activated, immobilized enzyme molecules are phosphomonoester derivatives of redox mediators (e.g., *p*-aminophenylphosphate) for metallophosphatases, phosphodiester derivatives of redox mediators (e.g., *p*-aminophenyl thymidine-5'-phosphate) for venom exonuclease (phosphodiesterase I), amino acid derivatives of redox mediators (e.g., L-alanine-*p*-aminophenol) for metalloaminopeptidases, and galactoside derivatives of redox mediators (e.g., *p*-aminophenyl-β-D-galactopyranoside) for β-galactosidase.

VI.3.1. Generation of redox mediators by activated immobilized enzyme

VI.3.1.1. Generation of redox mediators by activated immobilized alkaline phosphatase

If alkaline phosphatase (from *E. coli*) is the enzyme to be activated in the present invention, electrochemically inactive *p*-aminophenylphosphate may be used as substrate which is convertible to electrochemically active *p*-aminophenol. A 1 mM solution of the substrate, dissolved in 500 mM Tris-HCl, pH 8.0, is proper.

VI.3.1.2. Generation of redox mediators by activated immobilized _-galactosidase

If M15 β-galactosidase is the enzyme to be activated in the present invention, electrochemically inactive *p*-aminophenyl-β-D-galactopyranoside may be used as substrate which is convertible into electrochemically active *p*-aminophenol. A 2.5 mM solution of the substrate, dissolved in 100 mM sodium phosphate, 1 mM $MgCl_2$, 100 mM β-mercaptoethanol, pH 7.5, is proper.

VI.3.1.3. Generation of redox mediators by activated immobilized phosphodiesterase I

If venom exonuclease (phosphodiesterase I) is the enzyme to be activated in the present invention, electrochemically inactive *p*-aminophenyl thymidine-5'-phosphate may be used as substrate which is convertible to electrochemically active *p*-aminophenol. A 0.5 mM solution of the substrate, dissolved in 100 mM Tris-HCl, 15 mM $MgCl_2$, 100 mM NaCl, pH 8.9, is proper.

VI.3.2. Quantification of generated redox mediators

A closely spaced array of thin film nobel metal electrodes is utilized to quantify generated redox mediators as described by Wollenberger, U., Paeschke, M., and Hintsche, R. (Analyst 119, 1245, 1994). The microelectrodes consist of four pairs of microbands arranged on one silicon chip. Each finger electrode is 900 μm long and 3.2 μm wide. The gaps between the finger electrodes are 0.8 μm. The silicon chip is encapsulated by a 400 nm thick plasma-enhanced chemical vapour deposition (PECVD) SiO_2 layer, excluding the electrode area and the connecting pads. The chip sensor is electrically connected by means of gold wires bonded to the pads. These bonds are covered with silicon rubber resin.

Electrochemical measurements are carried out at 25 °C in 66 mM potassium phosphate / sodium phosphate, 100 mM KCl, pH 7.0, using an Ag-AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN, USA). A custom-made

multipotentiostat is used to control the potential of the electrodes independently. The current response of the individual electrodes is separately preamplified after current-to-voltage conversion and sent to a data acquisition board. Cyclic voltammograms are ontained with an Auto-Lab PSTAT 10 electrochemical analyzer (ECO Chemie, Utrecht, The Netherlands). In order to define the optimum potentials for oxidation and reduction of suitable mediators, cyclic and hydrodynamic voltammograms are recorded.

VI.4 IMMOBILIZATION OF INACTIVE ENZYME MOLECULES

VI.4.1. Immobilization of apometalloenzymes onto carbonyldiimidazole-activated beaded agarose

The preparation of carbonyldiimidazole (CDI)-activated cross-linked beaded agarose is performed as described by Wilchek, M. et al., (Meth. Enzyol. 104, 3, 1984). Cross-linked Sepharose 6B (3 g of moist cake) is washed sequentially with 20 ml each of water, dioxane-water (3: 7), dioxane-water (7: 3), and dioxane and ist suspended in 5 ml of dioxane. 1,1'-Carbonyldiimidazole (120 mg) is added, and the suspension is shaken at room temperature. After 15 min, the suspension is washed with 100 ml dioxane and used immediately. Alternatively, the matrix may be stored in dioxane under anhydrous conditions.

Prior to coupling of the apometalloenzyme, the CDI-activated cross-linked agarose beads are filtered and washed quickly with 0.1 M sodium bicarbonate, pH 8.5. Thereafter, the moist beads are resuspended in 0.1 M sodium bicarbonate, pH 8.5, containing the apometalloenzyme to be immobilized. After 20 hrs at 4 °C with gentle agitation, the residue is filtered and washed with 0.1 M $NaHCO_3$ and with distilled water. Excess active groups are removed by reaction for 1 hr at room temperature with either 0.1 M NH_4OH or 0.1 M ethanolamine at pH 9. All steps of the coupling procedure are performed in the presence of a chelating agent (e.g., 2 mM 1,10-phenanthroline) to prevent contamination of the apometalloenzyme by adventitious metal ions.

VI.4.2. Immobilization of apometalloenzymes onto tresyl- or tosyl-activated beaded agarose

The preparation of tresylated or tosylated cross-linked beaded agarose is performed as described by Nilsson, K. et al., (Meth. Enzymol. 104, 56, 1984). Sepharose 4B or CL-6B (10 g, wet) is washed successively with 100 ml of each of the following: 30: 70 and 70: 30 of acetone: water (v/v), twice with acetone, and three times with dry acetone (dried with a molecular sieve overnight) using 1 liter of acetone per 35 g of

Sepharose 4B or sepharose CL-6B, respectively. The gel is then transferred to a dried beaker. For tresyl-activation, the beaker containing 3 ml of dry acetone and 150 µl of dry pyridine (dried with a molecular sieve). During magnetic stirring, 100 µl of tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) (Fluka AG, Buchs, Switzerland) is added dropwise. After 10 min at room temperature, the gel is washed twice with 100 ml of each of the following: acetone, 30: 70 of 5 mM HCl: acetone, 50: 50 of 5 mM HCl: acetone, 70: 30 of 5 mM HCl: acetone, and 1 mM HCl. For tosyl-activation, the beaker contains 0,6 g of tosyl chloride (p-toluene sulfonyl chloride) dissolved in 3 ml of dry acetone. After addition of 1 ml dry pyridine (dried with a molecular sieve), the reaction is continued for 1 hr at room temperature with continuous magnetic stirring. The activated gel is washed as described for the tresylated gel. The tresylated or tosylated cross-linked agarose beads are stored at 4 °C until used. The reactivity of Sepharose tresyl groups is very high, allowing a 75 to 100% coupling yield of thiol- or amino-containing molecules within 1 hr at pH 7.5 in the cold. Thiols and primary amino groups are the most reactive nucleophiles with sulfonate esters on gels, thiols showing the highest reactivity.

Prior to coupling of the apometalloenzyme, the tresyl-activated cross-linked agarose beads are washed quickly with cold 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2. Thereafter, the moist agarose beads (1 g) are resuspended in 1 ml of 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2, containing the apometalloenzyme to be immobilized. After 20 hrs at 4 °C with gentle agitation, the gel is treated with 0.2 M Tris-HCl, pH 8.5, for 5 hrs at room temperature, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water. All steps of the coupling procedure are performed in the presence of a chelating agent (e.g., 2 mM 1,10-phenanthroline) to prevent contamination of the apometalloenzyme by adventitious metal ions.

Prior to coupling of the apometalloenzyme, the tosyl-activated cross-linked agarose beads are washed quickly with cold 0.25 M $NaHCO_3$ at pH 10.5. Sepharose tosyl groups require a pH of 9 to 10.5 for efficient coupling of amine-containing molecules. Thereafter, the moist agarose beads (0.7 g) are resuspended in 1 ml of 0.25 M $NaHCO_3$, pH 10.5, containing the apometalloenzyme to be immobilized. After 20 hrs at 40 °C with gentle agitation, the gel is treated with 0.8 M mercaptoethanol, pH 10, for 15 hrs at 40 °C, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water. All steps of the coupling procedure are performed in the presence of a chelating agent (e.g., 2 mM 1,10-phenanthroline) to prevent contamination of the apometalloenzyme by adventitious metal ions.

VI.4.3. Immobilization of apometalloenzymes onto aldehyde-activated beaded agarose

The preparation of aldehyde-containing cross-linked beaded agarose is performed as described by Porath, J., and Axén, R. (Meth. Enzymol. 44, 19, 1976). Sepharose CL-4B (10 g, wet) is suspended in 20 ml of water containing 200 mg of sodium metaperiodate. The temperature is increased to 45 °C over a 20 min period and maintained at this temperature for another 100 min under gentle stirring. Thereafter, the oxidized agarose beads are washed with water.

The degree of aldehyde formation may be assessed by aldehyde-mediated reduction of Cu^{2+} to Cu^{+} which can be detected using the bicinchoninic acid (BCA) reagent (Pierce Chemical Company, Rockford, IL, USA) as described by Smith, P.K., Anal. Biochem. 150, 76, 1985. The formation of Cu^{+} is in direct proportion to the amount of aldehydes present in the polymer. BCA forms a purple-colored complex with Cu^{+} which can be measured at 562 nm.

For coupling of the apometalloenzyme, polyaldehyde-derivatized Sepharose CL-4B is suspended in 0.1 M sodium bicarbonate, 0.15 M NaCl, pH 8.5, and mixed with apometalloenzyme dissolved in 0.1 M sodium bicarbonate, 0.15 M NaCl, pH 8.5. In a fume hood, to each ml of this mixture 0.2 ml of 1 M sodium cyanoborohydride is added and the reaction mixture is incubated for at least 6 hrs at room temperature. To block remaining aldehydes, 0.2 ml of 1 M Tris-HCl, pH 8, is added to each ml of the reaction mixture, and after an additional 2 hrs at room temperature, the Sepharose beads are washed with water. All steps of the coupling procedure are performed in the presence of a chelating agent (e.g., 2 mM 1,10-phenanthroline) to prevent contamination of the apometalloenzyme by adventitious metal ions.

VI.4.4. Immobilization of apometalloenzymes onto mercapto-activated beaded agarose

Prior to coupling of a particular apometalloenzyme to mercapto-activated cross-linked agarose beads, the correponding active metalloenzyme is derivatized with pyridyl disulfide residues using SPDP (N-succinimidyl 3-(2-pyridyldithio) propionate; Pierce Chemical Company, Rockford, IL, USA). To 1.0 ml of 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, containing the metalloenzyme, a 5 to 20-fold molar excess of SPDP in DMF is added. After incubation for 30 min at room temperature, the solution is subjected to gel filtration on Sephadex G-25 (Pharmacia) equilibrated with 0.1 M sodium acetate, 0.15 M NaCl, pH 4.5. At this point, the

derivative is stable and may be stored. The degree of substitution can be determined with an aliquot of the purified SPDP-derivatized enzyme at a known protein concentration by measurement of the OD_{343} (resulting from the release of pyridine-2-thione; extinction coefficient of 8,080 M^{-1} cm^{-1}) after the addition of dithiothreitol (DTT) to a final concentration of 50 mM. Thereafter, the pyridyl disulfide-derivatized metalloenzyme is used for the preparation of pyridyl disulfide-derivatized apometalloenzyme as described in section VIII.1.1.

The preparation of mercapto-activated cross-linked agarose beads is performed as described by Porath, J. et al. (Meth. Enzymol. 44, 19, 1976). Sepharose 6B (30 g of moist cake) is washed with water, suction-dried, suspended in 24 ml of 1 M NaOH followed by dropwise addition of epichlorohydrin over 15 min at room temperature with stirring (0.75 ml of epichlorohydrin is added for a degree of subsitution of approximately 50 μmol of SH-groups per gram beads; 4.5 ml of epichlorohydrin is added for a degree of subsitution of approximately 700 μmol of SH-groups per gram beads). The suspension is then incubated with continuous stirring for 2 hrs at 60 °C. The product is washed with water and with 0.5 M sodium phosphate buffer, pH 6.25. After suction-drying and resuspension in 30 ml of 0.5 M sodium phosphate buffer, pH 6.25, 30 ml of 2 M sodium thiosulfate are added to the beads, followed by stirring for 6 hrs at room temperature. The alkyl thiosulfate ester obtained is washed with water, suspended in 60 ml of 0.1 M sodium bicarbonate, and reduced by the addition of 50 ml of dithiothreitol (8 mg/ml) containing 1 mM EDTA. The resulting mercapto beads are washed with 300 ml of 0.1 M sodium bicarbonate, 1.0 M NaCl, 1 mM EDTA, and then with 100 ml of 1 mM EDTA. The mercapto beads are stored in 10 mM deaerated sodium acetate, 1 mM EDTA, pH 4.

Prior to coupling of the pyridyl disulfide-derivatized apometalloenzyme,the mercapto-activated cross-linked agarose beads are filtered and washed quickly with 50 mM sodium phosphate, 100 mM NaCl, 2 mM EDTA, pH 7.5. Thereafter, the cake is suspended in 50 mM sodium phosphate, 100 mM NaCl, 2 mM EDTA, pH 7.5, containing the pyridyl disulfide-derivatized apometalloenzyme to be immobilized. After 20 hrs at room temperature with gentle agitation, the residue is filtered and washed with 50 mM sodium phosphate, 100 mM NaCl, 2 mM EDTA, pH 7.5, and with distilled water. Excess active groups can be removed by reaction with iodoacetate.

VI.5. IMMOBILIZATION OF CAPTURE MOLECULES

VI.5.1. Activation of capture oligonucleotides

VI.5.1.1. Synthesis of 5'-amine derivatives of oligonucleotides

Method A. Covalent attachment of an amine terminal spacer molecule to the 5'-phosphate of oligonucleotides according to method A is performed via formation of a phosphorimidazolide intermediate in a carbodiimide reaction (based on the method of Ghosh, S.S. et al., Anal. Biochem. 178, 43, 1989). The formation of a phosphorimidazolide intermediate provides better reactivity towards amine nucleophiles than the carbodiimide phosphosdiester intermediate if carboddimide is used without added imidazole. The carbodiimide phosphodiester intermediate also is shorter-lived in aqueous conditions due to hydrolysis than the imidazolide.

The 5'-phosphate-containing oligonucleotide (7.5 -15 nmol in 7.5 μl) is added to a microfuge tube containing 1.25 mg of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride; Piece Chemical Company, Rockford, IL, USA). Immediately, 5 μl of 0.25 M bis-hydrazide compound (e.g., carbohydrazide or adipic acid dihydrazide) dissolved in 0.1 M imidazole, pH 6, is added. The reaction mixture is vortexed and centrifuged in a microcentrifuge at maximal rpm for 5 min. Thereafter, an additional 20 μl of 0.1 M imidazole, pH 6, is added and the reaction mixture incubated for another 30 min at room temperature. The hydrazide-labeled oligonucleotide is purified by gel filtration on Sephadex G-25 using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

For derivatization of 5'-phosphate-containing oligonucleotides with diamine spacer molecules (e.g., ethylene diamine or 1,6-diaminohexane) the same experimental procedue is used. The diamine compound is dissolved at a concentration of 0.25 M in 0.1 M imidazole, pH 6, and 5 μl of this soluition is added to the reaction mixture.

Method B. Using method B, the desired oligonucleotide is prepared using automated standard solid-phase phosphoramidite techniques on a scale of about 1 μmol of bound first nucleoside. The solid-phase synthesis protocol includes a) removal of dimethoxytrityl groups with 3% dichloroacetic acid in 1,2-dichloroethane, b) coupling of the incoming nucleoside 3'-methoxymorpholinophosphite with tetrazole, c) iodine oxidation of the intermediate phosphite to a phosphate, and d) a capping step with acetic anhydride. After synthesis of the required sequence, an extra round of synthesis is carried out using 25 μmol of N-monomethoxytrityl O-methoxydiiso propylaminophosphinyl 3-aminopropan (1) ol (prepared according to Connolly, B.A. Nucleic Acids Res. 15, 3131, 1987) and 60 μmol of tetrazole.

Thereafter, the methyl phosphate protecting groups are removed with thiophenol and cleavage from the resin together with deblocking of the base protecting groups is effected with ammonia. The crude monomethoxytrityl protected oligonucleotide is purified by C-18 reverse phase HPLC (flow rate of 1 ml/min) using a gradient of 0.1 M triethylammonium acetate, pH 6.5, containing 5% CH_3CN (buffer A) and 0.1 M triethylammonium acetate, pH 6.5, containing 65% CH_3CN (buffer B) (25% B at t = 0 min ; 75% B at t = 20 min). After removal of the solvent by evaporation, the purified monomethoxytrityl protected oligonucleotide is dissolved in 2 ml of 80% acetic acid and incubated for two hours. Thereafter, the acetic acid is removed by evaporation and the detritylated amino-containing oligonucleotide is is redissolved in a small volume of water.

VI.5.1.2. Synthesis of 5'-sulfhydryl derivatives of oligonucleotides

Method A. For the derivatization of 5'-phosphate-containing oligonucleotides with a terminal sulfhydryl group according to method A (based on a procedure of Ghosh, S.S., Kao, P.M., and Kwoh, D.Y. Bioconjugate Chem. 1, 71, 1990), the oligonucleotide (7.5 -15 nmol in 7.5 µl) is added to a microfuge tube containing 1.25 mg of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Piece Chemical Company, Rockford, IL, USA). Immediately, 5 µl of 0.25 M cystamine dissolved in 0.1 M imidazole, pH 6, is added. The reaction volume is mixed by vortexing and centrifuged in a microcentrifuge at maximal rpm for 5 min. Thereafter, an additional 20 µl of 0.1 M imidazole, pH 6, is added and the reaction mixture incubated for another 30 min at room temperature. For reduction of the cystamine-derivatized oligonucleotide, 20 µl of 1 M dithiothreitol is added. Thereby, 2-mercaptoethylamine is released from the cystamine modification site and a terminal free sulfhydryl group is created at the 5'-position of the oligonucleotide. After 15 min at room temperature, the sulfhydryl-derivatized oligonucleotide is purified by gel filtration on Sephadex G-25 using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

Method B. Using method B, the desired oligonucleotide is prepared by automated standard solid-phase phosphoramidite techniques on a scale of about 2.5 µmol of bound first nucleoside. The solid-phase synthesis protocol includes a) removal of dimethoxytrityl groups with 3% dichloroacetic acid in 1,2-dichloroethane, b) coupling of the incoming nucleoside 3'-methoxymorpholinophosphite with tetrazole, c) iodine oxidation of the intermediate phosphite to a phosphate, and d) a capping step with acetic anhydride. After synthesis of the required sequence, an extra round of synthesis is carried out using 25 µmol of an S-trityl-O-methoxymorpholinophosphite

derivative of 2-mercaptoethanol (dissolved in 0.3 ml of acetonitile and 0.2 ml of 1,2-dichloroethane), 3-mercaptopropan (1) ol (dissolved in 0.5 ml of acetonitrile), or 6-mercaptohexan (1) ol (dissolved in 0.5 ml of acetonitrile) and 75 μmol of tetrazole (dissolved in 0.5 ml of acetonitrile). The S-trityl-O-methoxymorpholinophosphite derivatives are prepared according to Connolly, B.A., and Rider, P. (Nucleic Acids Res. 13, 4485, 1985). Following coupling, the phosphite intermediate is oxidized by treatment with iodine. Thereafter, the phosphate protecting groups are removed with thiophenolate and cleavage from the resin together with deblocking of the base protecting groups is effected with ammonia. The crude monomethoxytrityl protected oligonucleotide is purified by C-18 reverse phase HPLC (flow rate of 1 ml/min) using a gradient of 0.1 M triethylammonium acetate, pH 6.5, containing 5% CH_3CN (buffer A) and 0.1 M triethylammonium acetate, pH 6.5, containing 65% CH_3CN (buffer B) (10% B at t = 0 min ; 80% B at t = 30 min; 100%B at t = 40 min). The purified S-trityl-containing oligonucleotide in 0.1 M triethylammonium acetate, pH 6.5, is treated with a five-fold molar excess of $AgNO_3$. After 30 min a seven-fold molar excess of dithiothreitol is added and after another 30 min the precipitated Ag^+ salt of dithiothreitol is removed by centrifugation. After reduction of the volume by rotary evaporation, the thiol-containing oligonucleotide is used immediately for further derivatization. Alternatively, the thiol-containing oligonucleotide can be stored frozen at -20 °C for months with no decomposition.

VI.5.1.3. Pyridyl disulfide modification of 5'-amine-containing oligonucleotides

Oligonucleotides that have been modified with an amine-terminal spacer molecule can be reacted further with the heterobifunctional cross-linking reagent SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate; Pierce Chemical Company, Rockford, IL, USA). Oligonucleotides derivatized with a terminal pyridyl disulfide residue then can be coupled with sulfhydryl-containing molecules, forming a disulfide bond. Reduction of the terminal pyridyl disulfide residue releases the pyridine-2-thione leaving group and generates a terminal sulhydryl group. This procedure allows conjugation of the 5'-thiolated oligonucleotide to sulfhydryl-reactive derivatives.

First, SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution). The amine-derivatized oligonucleotide is dissolved in 250 μl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 μl of the SPDP solution. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

To release the pyridine-2-thione leaving group and form the free sulfhydryl, the oligonucleotide derivative is mixed with 20 µl of 1 M dithiothreitol and incubated for 15 min at room temperature. If present in sufficient quantity, the release of pyridine-2-thione can be monitored by its characteristic absorbance at 343 nm ($\varepsilon = 8.08 \times 10^3$ M^{-1} cm^{-1}). The thiolated oligonucleotide is purified from ecxess dithiothreitol by dialysis or gel filtration using 50 mM sodium phosphate, 1 mM EDTA, pH 7.2. The thiolated oligonucleotide is used immediately for further coupling reactions to prevent sulfhydryl oxidation.

VI.5.1.4. Thioester modification of 5'-amine-containing oligonucleotides

The NHS ester of SATA (N-succinimidyl S-acetylthioacetate; Pierce Chemical Company, Rockford, IL, USA) introduces a thioester moiety. The acetyl protecting group can be removed by treatment with neutral hydroxylamine. The resulting terminal sulfhydryl group can be used for subsequent conjugation to thiol-reactive molecules. The advantage of using SATA over disulfide-containing thiolation reagents such as SPDP is that the introduction of sulfhydryl residues does not include the use of a disulfide reducing agent. The pyridiyl dithiol group resulting from an SPDP thiolation must be reduced with a reducing agent such as dithiothreitol to free the sulfhydryl group. With SATA, the sulfhydryl is freed by hydroxylamine, thus eliminating the need for removal of sulfhydryl reductants prior to a conjugation reaction.

First, SATA is dissolved at a concentration of 8 mg/ml in DMF. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 250 µl of the SATA solution. After reaction for 3 hours at 37 °C, excess reagents are removed from the modified oligonucleotide by gel filtration.

To deprotect the thioacetyl group, 100 µl of 50 mM hydroxylamine hydrochloride, 2.5 mM EDTA, pH 7.5, is added and reacted for 2 hours at 37 °C. The sulfhydryl-containing oligonucleotide is used immediately for further reaction with a sulfhydryl-reactive molecule.

VI.5.1.5. Derivatization of oligonucleotides with an aldehyde function at the 5'-terminus

The cross-linking reagent SFB (succinimidyl *p*-formylbenzoate) can be used to add aldehyde groups to amine-containing oligonucleotides. First, SFB is dissolved at a concentration of 12.35 mg/ml in acetonitrile (makes a 50 mM solution). The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH

7.5, and mixed with 50 µl of the SFB solution. After reaction for 3 hours at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

VI.5.2. Coupling of activated capture oligonucleotides to spacer molecules

To increase the fexibility and, thereby, the reactivity of immobilized capture oligonucleotides, spacer molecules are introduced between the solid support and the immobilized capture oligonucleotides.

Method A. In method A, the heterobifunctional reagents LC-SPDP (N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate; Pierce Chemical Company, Rockford, IL, USA) or sulfo-LC-SPDP (Pierce Chemical Company, Rockford, IL, USA) are utilized as spacer molecules. The sulfo-NHS form of the cross-linker contains a negatively charged sulfonate group that provides water-solubility to the cross-linker.

LC-SPDP is dissolved at a concentration of 8.5 mg/ml in DMF (makes a 20 mM stock solution). If the water-soluble sulfo-LC-SPDP is used, a stock solution in water is prepared just prior to addition of an aliquot to the reaction, since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester. A 10 mM stock solution of sulfo-LC-SPDP is prepared by dissolving 5.2 mg/ ml water. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 µl of the LC-SPDP solution. If the water-soluble sulfo-LC-SPDP is used, 100 µl of the sulfo-LC-SPDP solution is added. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

Method B. In method B, water-soluble heterobifunctional derivatives of polyethylene glycol (PEG) are utilized as spacer molecules. Heterobifunctional PEG derivatives containing an amine-reactive N-hydroxysuccinimidyl (NHS) moiety and a sulfhydryl-reactive vinylsulfone (VS) moiety are especially useful, since the VS moiety is hydrolytically stable in aqueous media. At pH 7, the VS moiety reacts selectively with sulfhydryl groups. Reaction with amino groups proceeds at higher pH, but is still relatively slow.

NHS-PEG-VS (MW 3400; Shearwater Polymers Europe, Enschede, Netherlands) or NHS-PEG-VS (MW 2000; Shearwater Polymers Europe, Enschede, Netherlands) is dissolved in DMF at a concentration of 10 mM. The amine-derivatized

oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 100 µl of the NHS-PEG-VS solution. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

VI.5.3. Immobilization of activated capture oligonucleotides

For the immobilization of activated capture oligodeoxynucleotides, a variety of solid support systems providing different reactive residues is available. The examples describe the preparation of some suitable solid support systems and their application for immobilization of activated capture oligodeoxynucleotides. Each of the described procedures can be easily adjusted for the introduction of spacer molecules between the solid support and the immobilized capture oligonucleotides to increase the fexibility and, thereby, the reactivity of immobilized capture oligodeoxynucleotides. Furthermore, each of the described procedures allows to modify the density of immobilized capture oligonucleotides by co-immobilizing hydrophilic compounds capable of blocking immobilization sites for capture oligodeoxynucleotides (e.g., for amine-reactive solid supports: aminoethanol or monomethoxy-poly(ethylene glycol) containing a terminal amino group).

VI.5.3.1. Immobilization of activated capture oligodeoxynucleotides onto mercapto-activated beaded agarose

The preparation of mercapto-activated cross-linked agarose beads is performed as described by Porath, J., and Axen, R. (Meth. Enzymol. 44, 19, 1976) and detailed under item VI.4.4.

Prior to coupling of 5'-pyridyl disulfide derivatized oligodeoxynucleotides, the mercapto-activated cross-linked agarose beads are filtered and washed quickly with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5. Thereafter, the cake is suspended in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, containing the 5'-pyridyl disulfide-derivatized oligodeoxynucleotide to be immobilized. After 20 hrs at room temperature with gentle agitation, the residue is filtered and washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, and with distilled water. Excess active groups can be removed by reaction with iodoacetate.

VI.5.3.2. Immobilization of activated capture oligodeoxynucleotides onto pyridyl disulfide-activated beaded agarose

Mercapto-activated cross-linked agarose beads (section VI.5.3.1) are washed with water and a solution of 50% acetone-water. Thereafter, the beads are resuspended in 50% aqueous acetone and mixed with 100 mg 2,2'-dipyridyl disulfide dissolved in 50% aqueous acetone. After 30 min at room temperature, the beads are washed with 50% aqueous acetone containing 1 mM EDTA.

Prior to coupling of 5'-sulfhydryl-derivatized oligodeoxynucleotides, the pyridyl disulfide-activated cross-linked agarose beads are filtered and washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5. Thereafter, the cake is suspended in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, containing the 5'-sulfhydryl-derivatized oligodeoxynucleotide to be immobilized. After 20 hrs at room temperature with gentle agitation, the residue is filtered and washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, and with distilled water.

VI.5.3.3. Immobilization of activated capture oligodeoxynucleotides onto carbonyldiimidazole-activated beaded agarose

The preparation of carbonyldiimidazole (CDI)-activated cross-linked beaded agarose is performed as described by Wilchek, M. et al., (Meth. Enzyol. 104, 3, 1984). Cross-linked Sepharose 6B (3 g of moist cake) is washed sequentially with 20 ml each of water, dioxane-water (3: 7), dioxane-water (7: 3), and dioxane and ist suspended in 5 ml of dioxane. 1,1'-Carbonyldiimidazole (120 mg) is added, and the suspension is shaken at room temperature. After 15 min, the suspension is washed with 100 ml dioxane and used immediately. Alternatively, the matrix may be stored in dioxane under anhydrous conditions.

Prior to coupling of 5'-amine derivatized oligodeoxynucleotides, the CDI-activated cross-linked agarose beads are filtered and washed quickly with 0.1 M sodium bicarbonate, pH 8.5. Thereafter, the moist beads are resuspended in 0.1 M sodium bicarbonate, pH 8.5, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 4 °C with gentle agitation, the residue is filtered and washed with 0.1 M $NaHCO_3$ and with distilled water. Excess active groups are removed by reaction for 1 hr at room temperature with either 0.1 M NH_4OH or 0.1 M ethanolamine at pH 9.

VI.5.3.4. Immobilization of activated capture oligodeoxynucleotides onto tresyl-activated beaded agarose

The preparation of tresylated cross-linked beaded agarose is performed as described by Nilsson, K. et al. (Meth. Enzymol. 104, 56, 1984) and further detailed under VI.4.2.

Prior to coupling of 5'-amine derivatized oligodeoxynucleotides, the tresyl-activated cross-linked agarose beads are washed quickly with cold 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2. Thereafter, the moist agarose beads (1 g) are resuspended in 1 ml of 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 4 °C with gentle agitation, the gel is treated with 0.2 M Tris-HCl, pH 8.5, for 5 hrs at room temperature, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water.

VI.5.3.5. Immobilization of activated capture oligodeoxynucleotides onto tosyl-activated beaded agarose

The preparation of tosylated cross-linked beaded agarose is performed as described by Nilsson, K. and Mosbach, K. (Meth. Enzymol. 104, 56, 1984) and further detailed under VI.4.2.

Prior to coupling of 5'-amine derivatized oligodeoxynucleotides, the tosyl-activated cross-linked agarose beads are washed quickly with cold 0.25 M $NaHCO_3$ at pH 10.5. Sepharose tosyl groups require a pH of 9 to 10.5 for efficient coupling of amine-containing molecules. Thereafter, the moist agarose beads (0.7 g) are resuspended in 1 ml of 0.25 M $NaHCO_3$, pH 10.5, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 40 °C with gentle agitation, the gel is treated with 0.8 M mercaptoethanol, pH 10, for 15 hrs at 40 °C, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water.

VI.5.3.6. Immobilization of activated capture oligodeoxynucleotides onto sulfhydryl-containing polyacrylhydrazidoagarose (PAHOS)

Sepharose beads derivatized with linear polyacrylic hydrazide (PAHOS) provide properties of Sepharose and polyacrylamide gels. The preparation of PAHOS is performed as described by by Wilchek, M., Miron, T., and Kohn, J. (Meth. Enzmyol. 104, 3, 1984). Washed Sepharose 4B (10 g) is suspended in 30 ml of freshly prepared 0.25 M sodium periodate. The suspension is slowly stirred at room

temperature for 3 hrs in the dark. The oxidized Sepharose is then washed with cold and resuspended in three volumes of aqueous polyacrylhydrazide solution (0.1 - 0.5%) prepared from polymethylacrylate and hydrazine hydrate as described (Wilchek, M., and Miron, T. Meth. Enzymol. 34, 72, 1974). After slow stirring for 16 hrs in the dark at room temperature, the beads are washed extensively with 0.1 M NaCl, and then reduced with 0.3 M sodium borohydride in 0.5 M Tris-HCl, pH 8, for 3 hrs at room temperature. The reduced gel is washed with water on a sintered-glass funnel and stored at 4 °C.

Sulfhydryl groups are introduced by treating the gel with N-acetylhomocysteine thiolactone. The thiolactone (1 g) is added to a cold suspension of 10 ml of PAHOS beads in 20 ml of 1 M $NaCO_3$. After slow stirring for 16 hrs at 4 °C, the product is washed extensively with water and 0.1 M NaCl.

Prior to coupling of 5'-pyridyl disulfide derivatized oligodeoxynucleotides, the sulfhydryl-activated PAHOS beads are washed quickly with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5. Thereafter, the beads are resuspended in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, containing the 5'-pyridyl disulfide-derivatized oligodeoxynucleotide to be immobilized. After incubation for 20 hrs at room temperature with gentle agitation, the beads are washed with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.5, and with distilled water. Excess active groups can be removed by reaction with iodoacetate.

VI.5.3.7. Immobilization of activated capture oligodeoxynucleotides onto tresyl-activated silica

Porous glass spheres (10 μm diameter, 500 Å pore diamter) (LiChrosphere Si, Altex Scientific, Inc., Berkeley, CA, USA) are treated with hot chromic acid cleaning solution follwed by rinsing with 100 ml per 1 g porous glass spheres of hot 1 M HNO_3 and water. Thereafter, the cleaned porous glass spheres are suspended in a 10% aqueous solution of glycidoxypropyl trimethoxysilane, degassed with ultrasonic vibration for 10 min, and kept for 2 hrs at 90 °C, during which time the pH is maintained at 3.0 with 1 M HCl. the glass sheres are then collected on a medium-porosity glass frit, rinsed with water and dried overnight at 105 °C in vacuo.

The preparation of tresylated porous glass spheres coated with a hydrophilic layer of glycerylpropyl groups is performed as described by Nilsson, K., and Mosbach, K. (Meth. Enzymol. 104, 56, 1984). The dried porous glass spheres (2 g) is washed

three times with 50 ml each of dry acetone (dried with a molecular sieve overnight). The spheres are then transferred to a dried beaker containing 2.5 ml of dry acetone and 130 µl of dry pyridine (dried with a molecular sieve). During magnetic stirring, 90 µl of tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) (Fluka AG, Buchs, Switzerland) is added dropwise. After 15 min at 0 °C, the spheres are washed twice with 50 ml of each of the following: acetone, 30: 70 of 5 mM HCl: acetone, 50: 50 of 5 mM HCl: acetone, 70: 30 of 5 mM HCl: acetone, and 1 mM HCl. For storage, the tresylated porous glass spheres are washed with water, 50: 50 (v/v) water: acetone, and acetone and dried.

For coupling of 5'-amine derivatized oligodeoxynucleotides, the tresylated porous glass spheres are resuspended in 1 ml of 0.2 M sodium phosphate, 0.5 M NaCl, pH 8.2, containing the 5'-amine-oligodeoxynucleotide derivative to be immobilized. After 20 hrs at 4 °C with gentle agitation, the spheres are treated with 0.2 M Tris-HCl, pH 8.5, for 5 hrs at room temperature, and washed with a) 0.2 M sodium acetate, 0.5 M NaCl, pH 3.5, b) 0.5 M NaCl, and c) distilled water.

VI.5.3.8. Immobilization of activated capture oligodeoxynucleotides onto aldehyde-activated polyester film

The polyester film is a polymer of glycerol and terephthalic acid (Gareware Chemical Co., India) which on partial acid hydrolysis and periodate oxidation provides aldehyde groups (Sarkar, M., and Mandal, C. J. Immunol. Meth. 83, 55, 1985). For partial hydrolysis, the film is hydrolyzed with 1 M H_2SO_4 for 6 hrs at 100 °C, washed with distilled water, and then treated with 0.1 M $NaIO_4$ for 24 hrs at 30 °C. After elimination of excess periodate by the addition of 0.1 M ethylene glycol, the film is washed with distilled water and stored at 4 oC in 50 mM sodium phosphate, 150 mM NaCl, pH 7.5.

For coupling of 5'-hydrazide derivatized oligodeoxynucleotides, the aldehyde-activated polyester film is incubated with the 5'-hydrazide-oligodeoxynucleotide derivative to be immobilized in 50 mM M sodium phosphate, 150 M NaCl, pH 7.5. After 16 hrs at room temperature with gentle agitation, the film is washed with 50 mM M sodium phosphate, 150 M NaCl, pH 7.5. To reduce the hydrazone bonds to more stable linkages, the film may be further incubated for 1 hr in the presence of 15 mM sodium cyanoborohydride.

VI.5.4. Immobilization of proteinaceous capture molecules

Immobilization of proteinaceous capture molecules or derivatives thereof may be accomplished non-covalently (for a review, see Messing, R.A. Meth. Enzymol. 44, 148, 1976) or covalently by any of the well-known chemical coupling methods (for a review, see Mosbach, K. (ed.) Meth. Enzymol. 44, 1976). For most applications covalent immobilization techniques are preferred since the non-specific adsorption process affects the reproducibility. Preferred support matrices and techniques for immobilization of proteinaceous capture molecules or derivatives thereof are identical with those described in section VIII.3. for immobilization of enzyme molecules.

VI.6. DERIVATIZATION OF INTERCALATING AGENTS WITH REACTIVE RESIDUES

VI.6.1. Derivatization of the daunosamine moiety of daunorubicin with 2-iminothiolane

The cyclic imidoester 2-iminothiolane reacts with amines to form a stable, positively charged linkage, while leaving a sulfhydryl group available for further coupling (Jue, R. et al., Biochemistry 17, 5399, 1978). Using this heterobifunctional reagent, the positive charge of the original amine of the daunosamine moiety is preserved and can bind electrostatically to the negatively charged phosphate groups of the DNA.

Daunorubicin hydrochloride (Sigma, St. Louis, MO, USA) is dissolved in dry DMF at a concentration of 4.5 mg/ml and treated with an excess of silver carbonate (Pietersz, G.A., Smyth, M.J., Farquhar, I., and McKenzie, C. In: Antibody-mediated delivery systems. (J.D. Rodwell, ed.) pp. 25-53, Marcel Dekker, New York, 1988). After shaking for 15 min, the mixture is centrifuged at 450 x g for 10 min. The supernatant is decanted and a slight molar excess of 2-iminothiolane (Pierce Chemical Company, Rockford, IL, USA) in DMF is added. After incubation of the reaction mixture for 3 hours at room temperature with stirring, DMF is evaporated and the residue dissolved in dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and finally dried and stored at 4 °C in the dark. The purity of 4-mercaptobutyrimidate-derivatized daunorubicin is analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.6.2. Derivatization of the daunosamine moiety of daunorubicin with LC-SPDP

The long-chain (LC) version of SPDP (N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate) provides an additional 6-aminohexanoate spacer group as compared to SPDP. As a consequence, LC-SPDP increases the flexibility of daunorubcin when conjugated to liposomal surfaces and reduces thereby potential steric hindrance problems.

The method of VI.6.1. is repeated, except that a slight molar excess of LC-SPDP (Pierce Chemical company, Rockford, IL, USA) is added instead of 2-iminothiolane. Analysis fo purity is detected as described for the 2-iminothiolane.

VI.6.3. Derivatization of the daunosamine moiety of daunorubicin with SIAXX

Since SIAXX (N-succinimidyl 6-[6-(((iodoacetyl)-amino)hexanoyl)amino]hexanoate) contains two aminohexanoate spacer groups, conjugates prepared with this reagent are connected by a spacer arm containing 16 atoms. Thus, SIAXX provides high flexibility to surface-attached daunorubicin molecules.

The method of VI.6.1. is repeated, except that a slight molar excess of SIAXX (Moleculare Probes, Eugene, OR, USA) is added instead of 2-iminothiolane. Analysis fo purity is detected as described for the 2-iminothiolane.

VI.6.4. Derivatization of the daunosamine moiety of daunorubicin with NHS-PEG-VS

NHS-PEG-VS, heterobifunctional poly(ethylene glycol) derivatives containing an amine-reactive N-hydroxysuccinimidyl (NHS) moiety and a sulfhydryl-reactive vinylsulfone (VS) moiety, are commercially available from Shearwater Polymers Europe (Enschede, The Netherlands). PEG-spacer molecules provide water-solubility and a high degree of flexibility.

Daunorubicin hydrochloride (Sigma, St. Louis, MO, USA) is dissolved in dry DMF at a concentration of 4.5 mg/ml and mixed with a slight molar excess of NHS-PG-VS (MW 2 kD or 3.4 kD) dissolved in DMF at a concentration of 10 mM. After incubation of the reaction mixture for 3 hours at room temperature with stirring, DMF is evaporated and the residue dissolved in dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and finally dried and stored at 4 °C in the dark. The

purity of VS-PG-derivatized daunorubicin is analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.6.5. Derivation of the N^2-position of actinomycin D with 1,3-propane diamine

Derivatization of the N^2-position of actinomycin D with 1,3-propanediamine is performed in three steps including the synthesis of 2-deamino-2-hydroxyactinomycin D, 2-deamino-2-chloroactinomycin D (both steps according to Moore, S., Kondo, M. et al., J. J. Med. Chem. 18, 1098, 1975), and N^2-(3'-aminopropyl) actinomycin D (according to Sengupta, S.K. et al., J. Med. Chem. 24, 1052, 1981).

a) Synthesis of 2-deamino-2-hydroxyactinomycin D. A solution of actinomycin D (1.2 g) in 10% HCl (300 ml) is heated for 4.5 hr at 60°C. On cooling, the solution is extracted with chloroform (3 x 200 ml). The chloroform solution is washed twice with water and saline, dried, and evaporated. The product is chromatographed on Sephadex LH-20 using 95% ethanol as eluent. The purity of 2-deamino-2-hydroxy actinomycin D is confirmed by TLC using silica gel plates and *sec*-BuOH: HCOOH: H_2O (75: 13.5: 11.5) as solvent system.

b) Synthesis of 2-deamino-2-chloroactinomycin D. 2-Deamino-2-hydroxy actinomycin D is dissolved in dry benzene (50 ml). First, chloranil (297 mg) is added, followed by freshly destilled thionyl chloride (8.8 ml). The reaction mixture is refluxed under anhydrous conditions for 25 min. After cooling, the solution is evaporated to dryness and reevaporated several times from benzene. The product is precipitated from benzene with cold hexane and separated by centrifugation. The purity of 2-deamino-2-chloroactinomycin D is confirmed by TLC using silica gel plates and *sec*-BuOH: HCOOH: H_2O (75: 13.5: 11.5) or EtOAc: acetone (2: 1) as solvent system.

c) Synthesis of N^2-(3'-aminopropyl)actinomycin D. 2-Deamino-2-chloro actinomycin D (11 mg) is dissolved in methylene chloride (4 ml) and mixed with 100 µl (140 equiv) of 1,3-propanediamine (over 99.9% pure). After stirring for 3 hr at 45 -50°C under N_2, the reaction mixture is diluted with 50 ml methylene chloride and extracted with water (3 x 10 ml). Thereafter, the organic solvent is removed by evaporation. The purity of N^2-(3'-aminopropyl)actinomycin D is confirmed by TLC using silica gel plates and EtOAc: acetone (2: 1) as solvent system.

VI.6.6. Derivatization of N^2-(3'-aminopropyl) actinomycin D with LC-SPDP

The long-chain (LC) version of SPDP (N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate) provides a propionamidohexanoate spacer group which increases the flexibility of actinomycin D when conjugated to liposomal surfaces.

N^2-(3'-Aminopropyl)actinomycin D is dissoved in dry DMF and mixed with a slight molar excess of LC-SPDP (Pierce Chemical Company, Rockford, IL, USA) in DMF. After incubation of the reaction mixture for 3 hours at room temperature with stirring, DMF is evaporated and the residue dissolved in dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and finally dried and stored at 4 °C in the dark. The purity ofLC-SPDP-derivatized daunorubicin is analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.6.7. Derivatization of N^2-(3'-aminopropyl) actinomycin D with SIAXX

SIAXX (N-succinimidyl 6-[6-(((iodoacetyl)-amino)hexanoyl)amino]hexanoate) contains two aminohexanoate spacer groups which provide high flexibility to surface-attached actinomycin D molecules.

The method of VI.6.6. is repeated, except that a slight molar excess of SIAXX (Moleculare Probes, Eugene, OR, USA) is added instead of N^2-(3'-Aminopropyl)actinomycin D . Analysis fo purity is detected as described for the N^2-(3'-Aminopropyl)actinomycin D .

VI.6.8. Derivatization of N^2-(3'-aminopropyl) actinomycin D with NHS-PEG-VS

NHS-PEG-VS, heterobifunctional poly(ethylene glycol) derivatives containing an amine-reactive N-hydroxysuccinimidyl (NHS) moiety and a sulfhydryl-reactive vinylsulfone (VS) moiety, are commercially available from Shearwater Polymers Europe (Enschede, The Netherlands). PEG-spacer molecules provide water-solubility and a high degree of flexibility.

N^2-(3'-Aminopropyl)actinomycin D is dissolved in dry DMF and mixed with a slight molar excess of NHS-PG-VS (MW 2 kD or 3.4 kD) dissolved in DMF at a concentration of 10 mM. After incubation of the reaction mixture for 3 hours at room temperature with stirring, DMF is evaporated and the residue dissolved in

dichloromethane. Diethyl ether is added until precipitation is completed. The precipitate is washed several times with diethyl ether/dichloromethane (2:1) and finally dried and stored at 4 °C in the dark. The purity of VS-PG-derivatized daunorubicin is analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) and n-butanol: acetic acid: water at a ratio of 4: 1: 5 as solvent.

VI.7. DERIVATIZATION OF OLIGONUCLEOTIDES WITH REACTIVE RESIDUES FOR ATTACHMENT TO AFFINITY LIPOSOMES

VI.7.1. Synthesis of 5'-amine derivatives of oligonucleotides

Method A. Covalent attachment of an amine terminal spacer molecule to the 5'-phosphate of oligonucleotides according to method A is performed via formation of a phosphorimidazolide intermediate in a carbodiimide reaction (based on the method of Ghosh, S.S., Kao, P.M., and Kwoh, D.Y. Anal. Biochem. 178, 43, 1989). The formation of a phosphorimidazolide intermediate provides better reactivity towards amine nucleophiles than the carbodiimide phosphosdiester intermediate if carboddimide is used without added imidazole. The carbodiimide phosphodiester intermediate also is shorter-lived in aqueous conditions due to hydrolysis than the imidazolide.

The 5'-phosphate-containing oligonucleotide (7.5 -15 nmol in 7.5 µl) is added to a microfuge tube containing 1.25 mg of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride; Pierce Chemical Company, Rockford, IL, USA). Immediately, 5 µl of 0.25 M bis-hydrazide compound (e.g., carbohydrazide or adipic acid dihydrazide) dissolved in 0.1 M imidazole, pH 6, is added. The reaction mixture is vortexed and centrifuged in a microcentrifuge at maximal rpm for 5 min. Thereafter, an additional 20 µl of 0.1 M imidazole, pH 6, is added and the reaction mixture incubated for another 30 min at room temperature. The hydrazide-labeled oligonucleotide is purified by gel filtration on Sephadex G-25 using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

For derivatization of 5'-phosphate-containing oligonucleotides with diamine spacer molecules (e.g., ethylene diamine or 1,6-diaminohexane) the same experimental procedue is used. The diamine compound is dissolved at a concentration of 0.25 M in 0.1 M imidazole, pH 6, and 5 µl of this soluition is added to the reaction mixture.

Method B. Using method B, the desired oligonucleotide is prepared using automated standard solid-phase phosphoramidite techniques on a scale of about 1 µmol of bound first nucleoside. The solid-phase synthesis protocol includes a)

removal of dimethoxytrityl groups with 3% dichloroacetic acid in 1,2-dichloroethane, b) coupling of the incoming nucleoside 3'-methoxymorpholinophosphite with tetrazole, c) iodine oxidation of the intermediate phosphite to a phosphate, and d) a capping step with acetic anhydride. After synthesis of the required sequence, an extra round of synthesis is carried out using 25 μmol of N-monomethoxytrityl O-methoxydiiso propylaminophosphinyl 3-aminopropan (1) ol (prepared according to Connolly, B.A. Nucleic Acids Res. 15, 3131, 1987) and 60 μmol of tetrazole. Thereafter, the methyl phosphate protecting groups are removed with thiophenol and cleavage from the resin together with deblocking of the base protecting groups is effected with ammonia. The crude monomethoxytrityl protected oligonucleotide is purified by C-18 reverse phase HPLC (flow rate of 1 ml/min) using a gradient of 0.1 M triethylammonium acetate, pH 6.5, containing 5% CH_3CN (buffer A) and 0.1 M triethylammonium acetate, pH 6.5, containing 65% CH_3CN (buffer B) (25% B at t = 0 min ; 75% B at t = 20 min). After removal of the solvent by evaporation, the purified monomethoxytrityl protected oligonucleotide is dissolved in 2 ml of 80% acetic acid and incubated for two hours. Thereafter, the acetic acid is removed by evaporation and the detritylated amino-containing oligonucleotide is is redissolved in a small volume of water.

VI.7.2. Synthesis of 5'-sulfhydryl derivatives of oligonucleotides

Method A. For the derivatization of 5'-phosphate-containing oligonucleotides with a terminal sulfhydryl group according to method A (based on a procedure of Ghosh, S.S. et al., Bioconjugate Chem. 1, 71, 1990), the oligonucleotide (7.5 -15 nmol in 7.5 μl) is added to a microfuge tube containing 1.25 mg of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Piece Chemical Company, Rockford, IL, USA). Immediately, 5 μl of 0.25 M cystamine dissolved in 0.1 M imidazole, pH 6, is added. The reaction volume is mixed by vortexing and centrifuged in a microcentrifuge at maximal rpm for 5 min. Thereafter, an additional 20 μl of 0.1 M imidazole, pH 6, is added and the reaction mixture incubated for another 30 min at room temperature. For reduction of the cystamine-derivatized oligonucleotide, 20 μl of 1 M dithiothreitol is added. Thereby, 2-mercaptoethylamine is released from the cystamine modification site and a terminal free sulfhydryl group is created at the 5'-position of the oligonucleotide. After 15 min at room temperature, the sulfhydryl-derivatized oligonucleotide is purified by gel filtration on Sephadex G-25 using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

Method B. Using method B, the desired oligonucleotide is prepared by automated standard solid-phase phosphoramidite techniques on a scale of about 2.5 μmol of bound first nucleoside. The solid-phase synthesis protocol includes a) removal of dimethoxytrityl groups with 3% dichloroacetic acid in 1,2-dichloroethane, b) coupling of the incoming nucleoside 3'-methoxymorpholinophosphite with tetrazole, c) iodine oxidation of the intermediate phosphite to a phosphate, and d) a capping step with acetic anhydride. After synthesis of the required sequence, an extra round of synthesis is carried out using 25 μmol of an S-trityl-O-methoxymorpholinophosphite derivative of 2-mercaptoethanol (dissolved in 0.3 ml of acetonitile and 0.2 ml of 1,2-dichloroethane), 3-mercaptopropan (1) ol (dissolved in 0.5 ml of acetonitrile), or 6-mercaptohexan (1) ol (dissolved in 0.5 ml of acetonitrile) and 75 μmol of tetrazole (dissolved in 0.5 ml of acetonitrile). The S-trityl-O-methoxymorpholinophosphite derivatives are prepared according to Connolly, B.A., and Rider, P. (Nucleic Acids Res. 13, 4485, 1985). Following coupling, the phosphite intermediate is oxidized by treatment with iodine. Thereafter, the phosphate protecting groups are removed with thiophenolate and cleavage from the resin together with deblocking of the base protecting groups is effected with ammonia. The crude monomethoxytrityl protected oligonucleotide is purified by C-18 reverse phase HPLC (flow rate of 1 ml/min) using a gradient of 0.1 M triethylammonium acetate, pH 6.5, containing 5% CH_3CN (buffer A) and 0.1 M triethylammonium acetate, pH 6.5, containing 65% CH_3CN (buffer B) (10% B at t = 0 min ; 80% B at t = 30 min; 100%B at t = 40 min). The purified S-trityl-containing oligonucleotide in 0.1 M triethylammonium acetate, pH 6.5, is treated with a five-fold molar excess of $AgNO_3$. After 30 min a seven-fold molar excess of dithiothreitol is added and after another 30 min the precipitated Ag^+ salt of dithiothreitol is removed by centrifugation. After reduction of the volume by rotary evaporation, the thiol-containing oligonucleotide is used immediately for further derivatization. Alternatively, the thiol-containing oligonucleotide can be stored frozen at -20 °C for months with no decomposition.

VI.7.3. Pyridyl disulfide modification of 5'-amine-containing oligonucleotides

Oligonucleotides that have been modified with an amine-terminal spacer molecule can be reacted further with the heterobifunctional cross-linking reagent SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate; Pierce Chemical Company, Rockford, IL, USA). Oligonucleotides derivatized with a terminal pyridyl disulfide residue then can be coupled with sulfhydryl-containing molecules, forming a disulfide bond. Reduction of the terminal pyridyl disulfide residue releases the pyridine-2-thione leaving group

and generates a terminal sulhydryl group. This procedure allows conjugation of the 5'-thiolated oligonucleotide to sulfhydryl-reactive derivatives.

First, SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution). The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 µl of the SPDP solution. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

To release the pyridine-2-thione leaving group and form the free sulfhydryl, the oligonucleotide derivative is mixed with 20 µl of 1 M dithiothreitol and incubated for 15 min at room temperature. If present in sufficient quantity, the release of pyridine-2-thione can be monitored by its characteristic absorbance at 343 nm ($\varepsilon = 8.08 \times 10^3$ M^{-1} cm^{-1}). The thiolated oligonucleotide is purified from ecxess dithiothreitol by dialysis or gel filtration using 50 mM sodium phosphate, 1 mM EDTA, pH 7.2. The thiolated oligonucleotide is used immediately for further coupling reactions to prevent sulfhydryl oxidation.

VI.7.4. Thioester modification of 5'-amine-containing oligonucleotides

The NHS ester of SATA (N-succinimidyl S-acetylthioacetate; Pierce Chemical Company, Rockford, IL, USA) introduces a thioester moiety. The acetyl protecting group can be removed by treatment with neutral hydroxylamine. The resulting terminal sulfhydryl group can be used for subsequent conjugation to thiol-reactive molecules. The advantage of using SATA over disulfide-containing thiolation reagents such as SPDP is that the introduction of sulfhydryl residues does not include the use of a disulfide reducing agent. The pyridiyl dithiol group resulting from an SPDP thiolation must be reduced with a reducing agent such as dithiothreitol to free the sulfhydryl group. With SATA, the sulfhydryl is freed by hydroxylamine, thus eliminating the need for removal of sulfhydryl reductants prior to a conjugation reaction.

First, SATA is dissolved at a concentration of 8 mg/ml in DMF. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 250 µl of the SATA solution. After reaction for 3 hours at 37 °C, excess reagents are removed from the modified oligonucleotide by gel filtration.

To deprotect the thioacetyl group, 100 µl of 50 mM hydroxylamine hydrochloride, 2.5 mM EDTA, pH 7.5, is added and reacted for 2 hours at 37 °C. The sulfhydryl-

containing oligonucleotide is used immediately for further reaction with a sulfhydryl-reactive molecule.

VI.7.5. Derivatization of oligonucleotides with an aldehyde function at the 5'-terminus

The cross-linking reagent SFB (succinimidyl *p*-formylbenzoate) can be used to add aldehyde groups to amine-containing oligonucleotides. First, SFB is dissolved at a concentration of 12.35 mg/ml in acetonitrile (makes a 50 mM solution). The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 µl of the SFB solution. After reaction for 3 hours at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

VI.7.6. Derivatization of activated oligonucleotides with spacer molecules

To increase the flexibility and, thereby, the reactivity of oligonucleotides attached to the surface of affinity liposomes, spacer molecules are introduced between the liposomal surface and the oligonucleotides.

Method A. In method A, the heterobifunctional reagents LC-SPDP (N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate; Pierce Chemical Company, Rockford, IL, USA) or sulfo-LC-SPDP (Pierce Chemical Company, Rockford, IL, USA) are utilized as spacer molecules. The sulfo-NHS form of the cross-linker contains a negatively charged sulfonate group that provides water-solubility to the cross-linker.

LC-SPDP is dissolved at a concentration of 8.5 mg/ml in DMF (makes a 20 mM stock solution). If the water-soluble sulfo-LC-SPDP is used, a stock solution in water is prepared just prior to addition of an aliquot to the reaction, since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester. A 10 mM stock solution of sulfo-LC-SPDP is prepared by dissolving 5.2 mg/ ml water. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 50 µl of the LC-SPDP solution. If the water-soluble sulfo-LC-SPDP is used, 100 µl of the sulfo-LC-SPDP solution is added. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

Method B. In method B, water-soluble heterobifunctional derivatives of polyethylene glycol (PEG) are utilized as spacer molecules. Heterobifunctional PEG derivatives containing an amine-reactive N-hydroxysuccinimidyl (NHS) moiety and a sulfhydryl-

reactive vinylsulfone (VS) moiety are especially useful, since the VS moiety is hydrolytically stable in aqueous media. At pH 7, the VS moiety reacts selectively with sulfhydryl groups. Reaction with amino groups proceeds at higher pH, but is still relatively slow.

NHS-PEG-VS (MW 2000; Shearwater Polymers Europe, Enschede, Netherlands) is dissolved in DMF at a concentration of 10 mM. The amine-derivatized oligonucleotide is dissolved in 250 µl of 50 mM sodium phosphate, pH 7.5, and mixed with 100 µl of the NHS-PEG-VS solution. After reaction for 1 hour at room temperature, excess reagents are removed from the modified oligonucleotide by gel filtration.

VI.8. DERIVATIZATION OF LIPID MOLECULES WITH REACTIVE RESIDUES AND AFFINITY COMPONENTS

VI.81. Activation of phosphatidyl ethanolamine with a pyridyl disulfide residue

Phosphatidyl ethanolamine (PE) (15 mg; 20 µmol) is dissolved in 2 ml of argon-purged, anhydrous methanol containing 20 µmol of triethylamine (TEA; 2 mg) and maintained over an argon or nitrogen atmosphere. After the addition of 30 µmol LC-SPDP (succinimidyl 6-[3-(2-pyridyldithio)propionamido] hexanoate; Pierce Chemical Company, Rockford, IL, USA) to the PE solution, the reaction mixture is mixed well and incubated for 2 h at room temperature while maintained under an argon atmosphere. Reaction progress is determined by thin-layer chromatography (TLC) using silica gel plates developed with a mixture of chloroform : methanol:water (by volume 65:25:4). The activated PE derivative (LC-PDP-PE) develops faster on TLC than the unmodified PE. After completion of the reaction, the methanol is removed from the reaction solution by rotary evaporation and the solid is redissolved in 5 ml of chloroform. The water-soluble reaction by-products are extracted twice from the chloroform with an equal volume of 1% NaCl. The LC-PDP-PE derivative is further purified by chromatography on a column of silicic acid as described by Martin, F.J., Heath, T.D., and New, R.R.C. (*In*: Liposomes, A Practical Approach, pp. 163-182, IRL Press, New York, 1990). Silicic acid (2 g) is dissolved in 10 ml of chloroform and poured into a syringe barrel containing a plug of glass wool at the bottom. The chloroform-dissolved lipids are applied on the silicic acid column, washed with 4 ml of chloroform, and then eluted with 4 ml each of the following series of chloroform:methanol mixtures: 4:0.25, 4:0.5, 4:0.75, and 4:1. Fractions of 2 ml are collected and monitored for the presence of purified LC-PDP-PE by TLC as described above. Finally, the chloroform is removed from the LC-PDP-PE by rotary

evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VI.8.2. Activation of phosphatidyl ethanolamine with a maleimide residue

Example VI.8.1. is repeated, but adding 50 mg SMPB (N-succinimidyl-4-(p-maleimidiphenyl) butyrate; Pierce Chemical Company, Rockford, IL, USA) to a 100 solution of 100 µmol PE in 5 ml methanol containing 100 µmol TEA. Further chromatographic purification of the MPB-PE derivative is performed on a column of silicic acid as described by Martin, F.J. et al. (Biochemistry 20, 4229, 1981). Silicic acid (2 g) is dissolved in 10 ml of chloroform and poured into a syringe barrel containing a plug of glass wool at the bottom. The chloroform-dissolved lipids are applied on the silicic acid column, washed with 4 ml of chloroform, and then eluted with 4 ml each of the following series of chloroform : methanol mixtures: 4 : 0.25, 4 : 0.5, 4 : 0.75, and 4 : 1. Fractions of 2 ml are collected and monitored for the presence of purified MPB-PE by TLC as described above. Finally, the chloroform is removed from the MPB-PE by rotary evaporation and the derivative is stored at -20°C under a nitrogen atmosphere until use.

VI.8.3. Activation of phosphatidyl ethanolamine with an iodoacetyl residue

Example VI.8.1. is repeated, but adding 30 µmol SIAXX (N-succinimidyl 6-[6-(((iodoacetyl)-amino)hexanoyl)amino]hexanoate; Pierce Chemical Company, Rockford, IL, USA) to the PE solution. Further chromatographic purification and storage of the IAXX-PE are performed as described for example VI.8.2.

VI.8.4. Activation of phosphatidyl ethanolamine with an aldehyde group

Example VI.8.1. is repeated, but adding 30 µmol SFPA (succinimidyl-*p*-formylphenoxyacetate; Molecular Probes, Eugene, OR, USA) to the PE solution. Further chromatographic of purification and storage of FPA-PE are performed as described for example VI.8.1.

VI.8.5. Activation of phosphatidyl ethanolamine with a thioester residue

Example VI.8.1. is repeated, but adding 30 µmol SATP (succinimidyl acetylthiopropionate; Molecular Probes, Eugene, OR, USA) to the PE solution. Further chromatographic of purification and storage of ATP-PE are performed as described for example VI.8.1.

VI.8.6. Derivatization of lipid molecules with affinity components

VI.8.6.1. Coupling of 2,4,6-trinitrobenzene sulfonic acid to phosphatidyl ethanolamine

Coupling of 2,4,6-trinitrobenzene sulfonic acid (TNBSA) to phosphatidyl ethanolamine (PE) is performed as described by van Houte, A.J., and Snippe, H. (In: Liposome Technology, vol. II (G. Gregoriadis, ed.) pp. 125-139, CRC Press, Inc., Boca Raton, FL, USA, 1984). TNBSA (10 mg, 26 μmol) is dissolved in 15 ml of 10 mM sodium phosphate, pH 8.5, and mixed with 15 ml of chloroform and 30 ml of methanol containing 13 μmol PE. After vigorous stirring for 2 hrs at room temperature, 15 ml of chloroform and 15 ml of 10 mM sodium phosphate, pH 8.5, are added. Phase separation is performed by centrifugation for 10 min at 1200 x g. The chloroform layer is washed once with 20 ml of 10 mM sodium phosphate, pH 8.5, and two times with 20 ml of distilled water. To the resulting chloroform layer 60 ml of methanol are added and the TNP-PE (dissolved in a methanol: chloroform mixture of 2:1) is stored under nitrogen at -20 °C.

VI.8.6.2. Coupling of NHS-LC-biotin to phosphatidyl ethanolamine

Coupling of NHS-LC-biotin to to phosphatidyl ethanolamine (PE) is performed as described by Bayer, E.A., and Wilchek, M. (In: Liposome Technology, vol. III (G. Gregoriadis, ed.) pp. 127-135, CRC Press, Inc., Boca Raton, FL, USA, 1984). PE (30 mg) is dissolved in 1 ml of a chloroform: methanol mixture (2: 1) containing 20 mg of NHS-LC-biotin (succinimidyl-6-(biotinamido) hexanoate; Pierce Chemical Company, Rockford, IL, USA). After the addition of 10 μl of triethylamine, the reaction mixture is incubated for 30 min at room temperature. The product (LC-biotin-PE) is purified by thin layer chromatography (TLC) on precoated silica 60 plates (Merck, Darmstadt, Germany) using a mixture of chloroform: methanol: water (80: 25: 2) for development. On the silica plates, the components are visualized with dimethylaminocinnamaldehyde, a biotin-specific reagent, or by exposure to iodine vapors. The product is scraped off the plate and extracted withchloroform: methanol (2:1). The solvent is evaporated under a stream of nitrogen, and the biotinylated lipid is stored at -20 °C.

VI.8.6.3. Coupling of 2-iminothiolane-derivatized daunorubicin to SPDP-derivatized phosphatidyl ethanolamine

2-Iminothiolane-derivatized daunorubicin (15 μmol) (section VI.6.1) is dissolved in 15 ml of degassed 10 mM sodium phosphate, 1 mM EDTA, pH 7.5, and mixed with 15 ml of chloroform and 15 ml of methanol containing 7 μmol SPDP-derivatized PE. After vigorous stirring for 3 hrs at room temperature, 15 ml of chloroform and 15 ml of 10 mM sodium phosphate, pH 7.5, are added. Phase separation is performed by

centrifugation for 10 min at 1200 x g. The chloroform layer is washed once with 20 ml of 10 mM sodium phosphate, pH 7.5, and two times with 20 ml of distilled water. To the resulting chloroform layer 60 ml of methanol are added and the daunorubicin-PE conjugate (dissolved in a methanol: chloroform mixture of 2:1) is stored under nitrogen at -20 °C.

VI.9. PREPARATION OF ENZYME ACTIVATOR-CONTAINING LIPOSOMES

VI.9.1. Preparation of liposomes containing pyridyl disulfide-derivatized phosphatidyl ethanolamine and encapsulated $ZnCl_2$

In the following examples, pyridyl disulfide-derivatized dipalmitoyl phosphatidyl ethanolamine (PD-DPPE) is utilized as reactive lipid derivative for subsequent coupling of affinity components to the liposomal surface. Other lipid derivatives including those containing a covalently attached affinity component (e.g., biotin-derivatized dipalmitoyl phosphatidyl ethanolamine) may be used as long as they are soluble in suitable organic solvents. For encapsulation, the enzyme activator $ZnCl_2$ is dissolved at a concentration of 5 M in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer in distilled water, pH 7.4.

VI.9.1.1. Preparation of MLV liposomes using pyridyl disulfide-derivatized PE

MLV liposomes containing encapsulated $ZnCl_2$ are prepared from a lipid mixture consisting, on a molar ratio basis, of dipalmitoyl phosphatidyl choline (PC), cholesterol, dipalmitoyl phosphatidyl glycerol (PG), and pyridyl disulfide-derivatized dipalmitoyl phosphatidyl ethanolamine (PD-DPPE) of 8:10:1:1. Using this percentage of cholesterol, the integrity of the liposomal bilayer will be stable up to a level of organic solvent addition of about 5%. This is important for subsequent coupling of affinity components added to the liposome suspension as a concentrated stock dilution in an organic solvent.

The lipid mixture solved in organic solvent (approximatey a total of 60 μmol/ml preparation) is pipetted into a round-bottom flask and then dried under reduced pressure at or close to its transition temperature (the highest transition temperature of any one lipid in the mixture is taken into consideration). Thereafter, the lipid mixture is hydrated with $ZnCl_2$ dissolved at a concentration of 5 M in 10 mM HEPES buffer, pH 7.4, by vortexing for at least 10 min in a water bath at or above the transition temperature of the lipid mixture. Next, the lipid mixture is mechanically shaken in the water bath for about 30 min and after another 30 min at room temperature (without skaking), the liposome mixture is filtered through 0.4 μm

nucleopore filter under nitrogen pressure. Sometimes it is necessary, to perform sequential filtering starting from 1.0, 0.6., and then 0.4 μm. The MLVs are dialyzed against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VI.9.1.2. Preparation of SUV liposomes using pyridyl disulfide-derivatized PE

SUVs are made from the MLV. After the first step in the MLV procedure (hydration by vortexing), the lipid mixture is transferred to a sonicating flask, equipped with a long neck and cap with in and out spouts for nitrogen. Nitrogen is flushed through the flask, then a light stream of nitrogen is left going into the tube and the outlet is closed. The flask is placed into a sonicator (e.g., Laboratory Supplies Company, Hicksville, NY, USA) at or above the highest transition temperature of any one lipid in the mixture. Sonication is performed for 30 to 60 min until the mixture appears opalescent. Thereafter, the SUVs are allowed to equilibrate for approximately 30 min at room temperature before uncaptured $ZnCl_2$ is removed by dialysis against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VI.9.1.3. Preparation of LUV liposomes using pyridyl disulfide-derivatized PE

For the preparation of LUV liposomes according to the reverse-phase evaporation technique of Szoka, F.C., and Paphadjopoulos, D. (Proc. Natl. Acad. Sci. USA 75, 4194, 1978), a lipid formulation consisting, on a molar ratio basis, of dipalmitoyl phosphatidyl choline (PC), cholesterol, dipalmitoyl phosphatidyl glycerol (PG), and pyridyl disulfide-derivatized dipalmitoyl phosphatidyl ethanolamine (PD-DPPE) of 8:10:1:1 is used. After mixing of the lipids solved in organic solvent, the solvent is evaporated to dryness on a rotary evaporator. The dried lipids are redissolved in isopropyl ether, freshly redistilled from sodium bisulfite, to a concentration of approximately 20 μmol/ml ether and transferred to a 50 ml screw-cap Erlenmeyer flask. Thereafter, the aqueous phase consisting of 5 M $ZnCl_2$ in 10 mM HEPES buffer in distilled water, pH 7.4, is added directly to the lipid solution in a ratio of 1:3 with ether. The flask is sealed with nitrogen, contents are mixed very well, and the mixture is sonicated at room temperature for at least 5 min until the mixture looks homogeneous. The organic phase is then removed by rotary evaporation under reduced pressure initially at about 450 mm Hg for small preparations and 550 mm Hg for larger preparations. When gel forms, vacuum is increased gradually to a maximum of 700 to 750 mm Hg. Foaming during this process can be eliminiated by

quick flushing of nitrogen into the flask. The temperature is also increased gradually from room temperature to about 37 °C towards the end of evaporation. At the end of the process, the residue is slightly less in volume than the original aqueous phae (at this stage, no odor of isopropyl ether should be detectable). Thereafter, the SUVs are allowed to equilibrate for approximately 30 to 60 min at room temperature, then extruded through 0.4 μm nucleopore filter under nitrogen pressure. Sometimes it is necessary, to perform sequential filtering starting from 1.0, 0.6., and then 0.4 μm. Uncaptured $ZnCl_2$ is removed by dialysis against 10 mM phosphate-buffered saline, pH 7.4 (at least 1: 100 volume; three or four changes; 1 hr each; then overnight at 4 °C). To obtain a more concentrated liposome suspension, it can be centrifuged at 100,000 x g (80 min at 20 °C).

VI.9.2. Preparation of temperature-sensitive liposomes containing phosphatidyl glycerol and encapsulated $ZnCl_2$

Since the goal is to produce affinity liposomes that undergo a sharp increase in release of encapsulated enzyme activators at T_m, it is necessary to minimize lipid-soluble contaminating substances. This condition is usually met by using synthetic lipids of greater than 99% fatty acid purity. In the described examples, the synthetic lipids dipalmitoyl phosphatidyl choline (DPPC) (T_m = 41 °C), dipalmitoyl phosphatidyl glycerol (DPPG) (T_m = 41 °C), and distearoyl phosphatidyl choline (DSPC) (T_m = 54 °C) are utilized. Incorporation of DPPG into the liposomal bilayer provides periodate-oxidizable vicinal hydroxyl groups which can be utilized to generate surface-attached aldehyde functions for covalent coupling of affinity components. For encapsulation, the enzyme activator $ZnCl_2$ is dissolved at a concentration of 5 M in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer in distilled water, pH 7.4.

VI.9.2.1. Preparation of temperature-sensitive SUV liposomes

For the preparation of temperature-sensitive SUV-affinity liposomes, the lipid formulation consists of DPPC, DPPG, and DSPC at a molar ratio of 6.5: 0.5: 3.0. The DSPC is added to counterbalance the lowering of T_m due to the small radius of curvature of SUV. The lipids are dried from benzene onto a glass vial under a stream of argon and lyophilized overnight. A 5 ml aliquot of the 5 M $ZnCl_2$ solution in 10 mM HEPES, pH 7.4, is warmed in a water bath to 50 °C and added to the vial containing approximately a total of 40 mg lipids, also at 50 °C. At the same temperature, the suspension is hydrated with repeated votex-mixing for about 15 min. Thereafter, the suspension is sonicated under argon to form SUV at 50 °C. After clarification of the suspension (usually within 5 min of sonication), the suspension is maintained above T_m for 15 min and centrifuged briefly at low speed

to remove insoluble material. Finally, non-encapsulated $ZnCl_2$ is removed by size exclusion chromatography on Sepharose 4B (Pharmacia) equilibrated in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4.

VI.9.2.2. Preparation of temperature-sensitive LUV liposomes

For the preparation of temperature-sensitive LUV-affinity liposomes according to the reverse-phase evaporation technique of Szoka, F.C., and Paphadjopoulos, D. (Proc. Natl. Acad. Sci. USA 75, 4194, 1978), a lipid formulation consisting of DPPC and DPPG at a molar ratio of 9: 1 is used. After mixing of the lipids solved in organic solvent, the solvent is evaporated to dryness on a rotary evaporator. The dried lipids (approximately a total of 125 mg) are redissolved in an organic phase consisting of 4 ml of chloroform and 8 ml of isopropyl ether, freshly washed with 10% sodium bisulfite. The mixture is transferred to a 50 ml screw-cap Erlenmeyer flask. The aqueous phase consists of 10 mM HEPES buffer in distilled water, pH 7.4, containing 5 M $ZnCl_2$. The organic phase (12 ml) and the aqueous phase (4 ml) are warmed to 50 °C and combined. The screw-cap Erlenmeyer flask is then filled with nitrogen gas and sealed with Teflon tape. The organic / aqueous mixture is placed in a cylindrical bath-type sonicator (e.g., Laboratory Supplies Company, Hicksville, N.Y., USA) filled with water at 45 to 50 °C, and sonicated for 5 min to form a milky, white, homogeneous emulsion. The emulsion is then transferred to a 125 ml tear-drop-shaped rotary evaporation flask. The water around the flask is kept at 50 °C. Upon lowering the pressure, the organic phase is drawn off. During this process the emulsion foams exuberantly and requires careful venting to adjust the pressure. After a period of 10 to 20 min, it is useful to add approximately 2 ml of additional HEPES buffer to replace the amount that is lost during the evaporation process. The procedure is finished when no foaming occurs at a pressure of approximately 150 mm Hg. The newly formed liposomes are allowed to anneal at 50 °C in a water bath for 30 min or longer. LUV affinity liposomes of well-defined size are formed by extrusion of this suspension through polycarbonate membranes above T_m. The liposomes are then rapidly cooled to room temperature in an ice bath and dialyzed over 24 hrs against two 1000 ml volumes of HEPES buffer to remove non-encapsulated $ZnCl_2$.

VI.9.2.3. Preparation of temperature-sensitive MLV liposomes

For the preparation of temperature-sensitive MLV-affinity liposomes, a lipid formulation consisting of DPPC and DPPG at a molar ratio of 9: 1 is used. After mixing of the lipids (aprproximately a total of 125 mg) solved in organic solvent, the solvent is evaporated to dryness on the wall of a 100 ml round-bottom fask. The

aqueous phase consists of 10 mM HEPES buffer in distilled water, pH 7.4, containing 5 M $ZnCl_2$. After heating of the aqueous phase to 50 °C, 4 ml are transferred to the flask containing the dried lipids, taking care to keep the flask above the T_m of the mixture. The flask is filled with nitrogen gas, closed with a glass stopper, and sealed with Teflon tape. Thereafter, the lipids are hydrated by repeated cycles of vortex-mixing for 15 sec followed by 1.5 min of incubation in a 50 °C water bath. The suspension is cycled 20 times in this manner and then allowed to anneal 30 min or longer in the 50 °C water bath. The liposomes are then rapidly cooled to room temperature in an ice bath and dialyzed over 24 hrs against two 1000 ml volumes of HEPES buffer to remove non-encapsulated $ZnCl_2$.

VI.10. DERIVATIZATION OF INTACT LIPOSOMES WITH REACTIVE RESIDUES

VI.10.1. Derivatization of intact liposomes with pyridyl disulfide residues

First, LUV, SUV, and MLV liposomes containing encapsulated enzyme activators are prepared from a lipid mixture consisting, on a molar ratio basis, of phosphatidyl choline (PC), cholesterol, phosphatidyl glycerol (PG), and phosphatidyl ethanolamine (PE) of 8:10:1:1 as described in section VI.9.1. Other lipid recipes may be used as long as they contain about this percentage of PE. In addition, if this level of cholesterol is maintained in the liposome, then the integrity of the bilayer will be stable up to a level of organic solvent addition of about 5%. This factor is important for adding an aliquot of the cross-linker to the liposome suspension as a concentrated stock dilution in an organic solvent. Any method of liposome formation may be used.

Derivatization of PE-liposomes with pyridyl disulfide residues can be performed with SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate; Pierce Chemical Company, Rockford, IL, USA), LC-SPDP (N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate; Pierce Chemical Company, Rockford, IL, USA), or sulfo-LC-SPDP (Pierce Chemical Company, Rockford, IL, USA). SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution). Alternatively, LC-SPDP is dissolved at a concentration of 8.5 mg/ml in DMF (also makes a 20 mM stock solution). If the water-soluble sulfo-LC-SPDP is used, a stock solution in water is prepared just prior to addition of an aliquot to the reaction, since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester. The sulfo-NHS form of the cross-linker contains a negatively charged sulfonate group that prevents the reagent from penetrating lipid bilayers. Thus, only the outer surfaces of

the liposomes are activated using sulfo-LC-SPDP. A 10 mM stock solution of sulfo-LC-SPDP is prepared by dissolving 5.2 mg/ml water.

To each milliliter of the liposome suspension to be modified, 25 - 50 µl of the stock solution of either SPDP or LC-SPDP in DMF is added. If sulfo-LC-SPDP is used, 50 - 100 µl of the stock solution in water is added to each milliliter of the liposome suspension. The reaction mixture is vortexed and reacted for 30 min at room temperature. Longer reaction times, even overnight, have no adverse effects. Finally, the modified liposomes are purified from reaction by-products by dialysis or gel filtration using Sephadex G-50. The derivatized liposomes may be used immediately for subsequent coupling reactions or stored in a lyophilized state in the presence of sorbitol as described by Friede, M., Van Regenmortel, M.H.V., and Schuber, F. (Anal. Biochem. 211, 117, 1993).

VI.10.2. Derivatization of intact liposomes with aldehyde functions

First, LUV, SUV, and MLV liposomes containing encapsulated enzyme activators are prepared from a lipid mixture consisting, on a molar ratio basis, of phosphatidyl choline (PC), cholesterol, phosphatidyl glycerol (PG), and other glycolipids of 8:10:1:1 as described in section VI.9.1. The other glycolipids that can be incorporated include phosphatidyl inositol, lactosylceramide, galactose cerebroside, or various gangliosides. Other liposome compositions may be used (e.g., recipes without cholesterol), as long as a periodate-oxidizable component containing vicinal hydroxyls is present. Any method of liposome formation may be used.

Temperature-sensitive SUV liposomes containing phosphatidyl glycerol and encapsulated enzyme activators are prepared from a lipid mixture consisting, on a molar ratio basis, of dipalmitoyl phosphatidyl choline (DPPC), dipalmitoyl phosphatidyl glycerol (DPPG), and distearoyl phosphatidyl choline (DSPC) at a molar ratio of 6.5: 0.5: 3.0 as described in section VIII.8.2.1. Temperature-sensitive LUV and MLV liposomes containing phosphatidyl glycerol and encapsulated enzyme activators are prepared from a lipid mixture consisting, on a molar ratio basis, of dipalmitoyl phosphatidyl choline (DPPC) and dipalmitoyl phosphatidyl glycerol (DPPG) at a molar ratio of 9: 1 as described in sections VI.9.2.2 and VI.9.2.3, respectively.

Sodium periodate is dissolved in water to a concentration of 0.6 M (128 mg of sodium periodate/ml of H_2O) and 200 µl of this stock solution is added with stirring to each milliliter of PG-liposomes (5 mg/ml) suspended in 20 mM sodium phosphate,

0.15 M NaCl, pH 7.4. After incubation at room temperature for 30 min in the dark, the oxidized liposomes are dialyzed against 20 mM sodium borate, o.15 M NaCl, pH 8.4, to remove unreacted periodate. This buffer is optimal for subsequent coupling with amine-containing affinity components such as antibodies. Alternatively, unreacted periodate can be removed by gel filtration using a column of Sephadex G-50. The periodate-oxidized liposomes may be used immediately for subsequent coupling reactions or stored in a lyophilized state in the presence of sorbitol as described by Friede, M. et al. (Anal. Biochem. 211, 117, 1993).

VI.11 COVALENT ATTACHMENT OF AFFINITY COMPONENTS TO INTACT ENZYME ACTIVATOR-CONTAINING LIPOSOMES

VI.11.1. Covalent attachment of intercalating agents to intact liposomes

VI.11.1.1. Coupling of vinylsulfone-derivatized daunorubicin to intact thioester-derivatized liposomes

A suspension (5 mg/ml) of acetylthiopropionate-derivatized liposomes (sections VIII.7.5. and VIII.8.) in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, is mixed with 0.5 M hydroxylamine hydrochloride in 50 mM sodium phosphate, 25 mM EDTA, pH 7.5 (100 µl per ml of liposome suspension). After 2 hrs at room temperature, the deacetylated liposomes are purified by gel filtration on Sephadex G-50 (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, 2.5 mM EDTA, pH 7.5, and used immediately to couple vinylsulfone-derivatized daunorubicin.

Daunorubicin derivatized at its daunosamine moiety with the heterobifunctional poly (ethylene glycol) (PEG) derivative NHS-PEG-VS (section VI.6.4.) is dissolved in DMF at a concentration of 10 mM. From this solution, 25 to 50 µl are added to each ml of purified sulfhydryl-containing liposomes. The bilayer of liposomes containing 50% cholesterol will be stable up to a level of organic solvent addition of about 5%. After 3 hrs at room temperature, the daunorubicin-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4.

VI.11.1.2. Coupling of sulfhydryl-derivatized daunorubicin to intact LC-SPDP-derivatized liposomes

Daunorubicin derivatized at its daunosamine moiety with 2-iminothiolane (section VI.6.1.) is dissolved in DMF at a concentration of 10 mM. From this solution, 25 to 50 µl are added to each ml of LC-SPDP-derivatized liposomes (sections VI.8.1. and VI.9.) suspended in 0.1 M sodium phosphate, 0.15 M NaCl, 2.5 mM EDTA, pH 7.5 (5mg lipid/ml). The bilayer of liposomes containing 50% cholesterol will be stable up

to a level of organic solvent addition of about 5%. After 3 hrs at room temperature, the daunorubicin-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4.

VI.1.2. Covalent attachment of oligonucleotides to intact liposomes

VI.11.2.1. Coupling of vinylsulfone-derivatized oligonucleotides to intact thioester-derivatized liposomes

Acetylthiopropionate-derivatized liposomes (sections VI.8.5. and VI.9.) suspended in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of 5 mg lipid/ml, are mixed with 0.5 M hydroxylamine hydrochloride in 50 mM sodium phosphate, 25 mM EDTA, pH 7.5 (100 µl per ml of liposome suspension). After 2 hrs at room temperature, the deacetylated liposomes are purified by gel filtration on Sephadex G-50 (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, 2.5 mM EDTA, pH 7.5, and used immediately to couple vinylsulfone (VS)-derivatized oligonucleotides prepared from 5'-amine-containing oligonucleotides (section VIII.4.1.1.) by reaction with the heterobifunctional poly (ethylene glycol) (PEG) derivative NHS-PEG-VS (section VIII.4.2.). An aliquot of 500 µl of 50 mM sodium phosphate, pH 7.5, containing the oligonucleotide-PEG-VS derivative at a concentration of 1 mM, is added to each ml of sulfhydryl-derivatized liposomes. After 3 hrs at room temperature, the oligonucleotide-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4.

VI.11.2.2. Coupling of sulfhydryl-derivatized oligonucleotides to intact LC-SPDP-derivatized liposomes

An aliquot of 500 µl of 10 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.2, containing 5'-sulfhydryl derivatives of oligonucleotides (section VI.5.1.2.) at a concentration of 1 mM, is added to each ml of LC-SPDP-derivatized liposomes (sections VI.8.1. and VI.9.) suspended in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5 (5mg lipid/ml). After 3 hrs at room temperature, the oligonucleotide-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

VIII.11.2.3. Coupling of hydrazide-derivatized oligonucleotides to intact aldehyde-derivatized liposomes

An aliquot of 500 µl of 10 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.2, containing oligonucleotide-5'-adipic acid hydrazide derivatives (section VI.5.1.1.) at a concentration of 1 mM, is added to each ml of aldehyde-derivatized liposomes

(section VI.10.2.) suspended in 20 mM sodium borate, 0.15 M NaCl, pH 8.4 (5mg lipid/ml). After 3 hrs stirring at room temperature, the oligonucleotide-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

For further stabilization of the formed hydrazone bonds, the oligonucleotide-derivatized liposomes may be treated with a reducing agent such as cyanoborohydride prior to purification by gel filtration. In a fume hood, 125 mg of sodium cyanoborohydride is dissolved in 1 ml water (makes a 2 M solution). This solution is allowed to sit for 30 min to eliminate most of the hydrogen-bubble evolution that could effect the liposome suspension. An aliquot of 10 µl of the cyanoborohydride solution is added to each ml of oligonucleotide-derivatized liposomes. After reaction at 4 °C overnight, the oligonucleotide-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

VI.11.3. Covalent attachment of proteinaceous affinity components to intact liposomes

Proteinaceous affinity components include antibodies (e.g., antibodies with specificity for captured antigens, antibodies with specificity for the subclass of captured antibodies, antibodies with specificity for double- and/or triple-stranded nucleic acids, or anti-hapten antibodies), enzymes (e.g., dihydrofolate reductase), and streptavidin (or avidin). Coupling of these proteinaceous affinity components to liposomes occasionally may include liposome aggregation. This may be due to the unique properties or concentration of the protein used, or it may be a result of liposome-to-liposome cross-linking during the conjugation process. Adjusting the amount of proteinaceous affinity component in the reaction mixture as well as the relative amount of reactive residues (e.g., pyridyl disulfide residues) attached to each proteinaceous affinity component may have to be done to solve an aggregation problem.

VI.11.3.1. Coupling of sulfhydryl-derivatized proteinaceous affinity components to intact LC-SPDP-derivatized liposomes

a) *Coupling of sulfhydryl-derivatized antibodies to intact LC-SPDP-derivatized liposomes.* To 1.0 ml of 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, containing 10 mg of IgG antibody, 16 µl of 20 mM LC-SPDP (N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate; Pierce Chemical Company, Rockford, IL, USA) in DMF (8.5 mg/ml) is added and mixed. This gives a molar ratio of 5 mol LC-

SPDP per mol of IgG. After incubation for 30 min at room temperature, the solution is subjected to gel filtration on Sephadex G-25 (Pharmacia) equilibrated with 0.1 M sodium acetate, 0.15 M NaCl, pH 4.5. At this point, the derivative is stable and may be stored. The degree of substitution can be determined with an aliquot of the purified LC-SPDP-derivatized antibody at a known protein concentration by measurement of the OD_{343} (resulting from the release of pyridine-2-thione; extinction coefficient of 8,080 $M^{-1}cm^{-1}$) after the addition of dithiothreitol (DTT) to a final concentration of 50 mM.

For reduction of the pyridyl disulfide residues, 0.5 M DTT is added to a final concentration of 50 mM to the purified LC-SPDP-derivatized antibody in 0.1 M sodium acetate, 0.15 M NaCl, pH 4.5. At pH 4.5, DTT reduces the pyridyl disulfide bonds, but not the intrinsic aliphatic disulfide bonds of antibodies. After 20 min at room temperature, the antibodies are separated from DTT by a second column passage on Sephadex G-25 (Pharmacia) equilibrated with 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4.

The purified sulfhydryl-derivatized antibodies are used immediately for coupling to LC-SPDP-derivatized liposomes (sections VI.8.1. and VI.9.) suspended at a concentration of 5 mg lipid/ml in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4. After the addition of an equal volume of sulfhydryl-derivatized antibodies (adjusted to 5 mg protein/ml) in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4, to the liposome suspension, the reaction mixture is stirred overnight at room temperature in a nitrogen or argon atmosphere to prevent lipid oxidation. Non-coupled antibodies are removed by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.4.

b) *Coupling of sulfhydryl-derivatized streptavidin to intact LC-SPDP-derivatized liposomes.*_To each ml of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing streptavidin at a concentration of 10 mg/ml, 25 µl of SATA (N-succinimidyl S-acetylthioacetate; Pierce Chemical Company, Rockford, IL, USA) dissolved in DMSO at a concentration of 13 mg/ml is added (for different concentrations of streptavidin in the reaction medium, the amount of SATA is adjusted proportionally, but not beyond 10% DMSO in the aqueous reaction medium). After 30 min at room temperature, the SATA-derivatized streptavidin is purified by gel filtration on Sephadex G-25 equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. At this point, the derivative is stable and may be stored.

For deprotection of the acetylated sulfhydryl groups, each ml of SATA-derivatized streptavidin is mixed with 100 µl of 0.5 M hydroxylamine in 0.1 M sodium phosphate, 10 mM EDTA, pH 7.2. After 2 hrs at room temperature, the thiolated streptavidin is purified by gel filtration on Sephadex G-25 equilibrated with 0.05 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.4.

Purified sulfhydryl-derivatized streptavidin is used immediately for coupling to LC-SPDP-derivatized liposomes (sections VI.8.1. and VI.9.) suspended at a concentration of 5 mg lipid/ml in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4. After the addition of an equal volume of sulfhydryl-derivatized streptavidin (adjusted to 5 mg protein/ml) in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4, to the liposome suspension, the reaction mixture is stirred overnight at room temperature in a nitrogen or argon atmosphere to prevent lipid oxidation. Non-coupled streptavidin is removed by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.4.

c) *Coupling of sulfhydryl-derivatized dihydrofolate reductase (DHFR) to intact LC-SPDP-derivatized liposomes.* To each ml of 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2, containing dihydrofolate reductase (from chicken liver; Sigma-Aldrich, Deisenhofen, Germany) at a concentration of 1 mg/ml, a 10-fold molar excess of sulfo-LC-SPDP (sulfo-N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate; Pierce Chemical Company, Rockford, IL, USA) (freshly dissolved in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 10 mM) is added. After 30 min at room temperature, the LC-SPDP-derivatized DHFR is purified by gel filtration on Sephadex G-25 equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. At this point, the derivative is stable and may be stored. The degree of substitution can be determined with an aliquot of the purified LC-SPDP-derivatized DHFR at a known protein concentration by measurement of the OD_{343} (resulting from the release of pyridine-2-thione; extinction coefficient of 8,080 M^{-1} cm^{-1}) after the addition of dithiothreitol (DTT) to a final concentration of 50 mM.

For reduction of the pyridyl disulfide residues, 0.5 M DTT is added to a final concentration of 50 mM to purified LC-SPDP-derivatized DHFR in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.4. Since DHFR contains no disulfide bonds, the incorporated pyridyl disulfide residues can be deprotected with DTT without detrimental effects to the enzyme. After 20 min at room temperature, the sulfhydryl-derivatized DHFR is separated from DTT by a second column passage on Sephadex

G-25 (Pharmacia) equilibrated with 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4.

Purified sulfhydryl-derivatized DHFR is used immediately for coupling to LC-SPDP-derivatized liposomes (sections VI.8.1. and VIII.9.) suspended at a concentration of 5 mg lipid/ml in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4. After addition of 2.5 ml of sulfhydryl-derivatized DHFR (adjusted to approximately 0.6 mg protein/ml) in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4, to each ml of the liposome suspension, the reaction mixture is stirred overnight at room temperature in a nitrogen or argon atmosphere to prevent lipid oxidation. Non-coupled DHFR is removed by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.4.

VI.11.3.2. Coupling of sulfhydryl-derivatized proteinaceous affinity components to intact SMPB-derivatized liposomes

Derivatization of proteinaceous affinity components with sulfhydryl groups is performed as described in VIII.10.3.1. In this example, sulfhydryl-derivatized IgG antibodies are coupled to intact SMPB-derivatized liposomes.

After purification by gel filtration (VI.11.3.1.) sulfhydryl-derivatized IgG antibodies are used immediately for coupling to SMPB-derivatized liposomes (sections VI.8.2. and VI.9.) suspended at a concentration of 5 mg lipid/ml in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4. After the addition of an equal volume of sulfhydryl-derivatized IgG antibodies (adjusted to 5 mg protein/ml) in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4, to the liposome suspension, the reaction mixture is stirred overnight at room temperature in a nitrogen or argon atmosphere to prevent lipid oxidation. Non-coupled antibodies are removed by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.4.

VI.11.3.3. Coupling of non-derivatized proteinaceous affinity components to intact aldehyde-derivatized liposomes

In this example, non-derivatized IgG antibodies are coupled to periodate-oxidized liposomes (Method A) or to glutaraldehyde-activated liposomes (Method B).

Method A. To each ml of periodate-oxidized liposomes (section VI.10.2.) suspended in 20 mM sodium borate, 0.15 M NaCl, pH 8.4, at a concentration of 5 mg lipid/ml, 500 µl of 20 mM sodium borate, 0.15 M NaCl, pH 8.4, containing non-derivatized IgG

antibodies at a concentration of 10 mg/ml, are added. After 2 hrs stirring at room temperature, the Schiff base interactions between the aldehydes on the liposomes and the amines on the antibodies are stabilized by reduction with cyanoborohydride. In a fume hood, 125 mg of sodium cyanoborohydride is dissolved in 1 ml water (makes a 2 M solution). This solution is allowed to sit for 30 min to eliminate most of the hydrogen-bubble evolution that could effect the liposome suspension. An aliquot of 10 µl of the cyanoborohydride solution is added to each ml of antibody-derivatized liposomes. After reaction at 4 °C overnight, the antibody-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

Method B. Phosphatidyl ethanolamine (PE) liposomes containing encapsulated enzyme activators are prepared as described in section VI.10.1. and suspended in 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.8, at a concentration of 5 mg lipid/ml. All buffers are degassed and bubbled with nitrogen or argon prior to use. After the addition of glutaraldehyde to a final concentration of 1.25%, the liposome suspension is reacted overnight at room temperature under a nitrogen atmosphere, and then purified from exess glutaraldehyde by gel filtration on Sephadex G-50 (Pharmacia) equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.8.

To each ml of purified glutaraldehyde-activated liposomes, 1 ml of 0.5 M sodium carbonate, pH 9.5, containing non-derivatized IgG antibodies at a concentration of 10 mg/ml, is added (ratio of 2 mg IgG antibody per mg lipid). After 3 hrs stirring at room temperature under a nitrogen atmosphere, any excess aldehydes and the Schiff base interactions between the aldehydes on the liposomes and the amines on the antibodies are reduced with cyanoborohydride. In a fume hood, 125 mg of sodium cyanoborohydride is dissolved in 1 ml water (makes a 2 M solution). This solution is allowed to sit for 30 min to eliminate most of the hydrogen-bubble evolution that could effect the liposome suspension. An aliquot of 10 µl of the cyanoborohydride solution is added to each ml of antibody-derivatized liposomes. After reaction at 4 °C overnight, the antibody-derivatized liposomes are purified by gel filtration on Sepharose 4B (Pharmacia) equlibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

VI.11.3.4. Coupling of hydrazide-derivatized proteinaceous affinity components to intact aldehyde-derivatized liposomes

In this example, streptavidin is derivatized with adipic acid dihydrazide according to a method of Bayer, E.A., Ben-Hur, H., and Wilchek, M. (Anal. Biochem. 161, 123,

1987). Adipic acid dihydrazide (160 mg) dissolved in 5 ml of 0.1 M sodium phosphate, pH 6 (some heating may be required during solubilization of the compound) is mixed with 50 mg streptavidin. To this solution, 160 mg of water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride is added and mixed thoroughly. After reaction for 4 hrs at room temperature, hydrazide-derivatized streptavidin is purified by gel filtration on Sephadex G-50 (Pharmacia) equilibrated with 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.8. Hydrazide-derivatized streptavidin may be stored as a freeze-dried preparation without loss of ativity.

Coupling of hydrazide-derivatized streptavidin to aldehyde-derivatized liposomes is performed as described in section VI.11.3.3. Stabilization by reduction with cyanoborohydride of the hydrazone linkages formed between the aldehydes on the liposomes and the hydrazide residues on streptavidin may be omitted, since hydrazone linkages provide a much higher stability than Schiff base interactions. However, the addition of a reductant during hydrazide/aldehyde reactions increases the efficiency and yield of the reaction.

VI.11.3.5. Incorporation of poteinaceous affinity component-palmitate conjugates into liposomal membranes

In this example, non-derivatized antibodies are coupled to palmitic acid derivatized with an N-hydroxysuccinimide ester, followed by incorporation of the antibody-palmitate conjugate into intact liposomes by detergent dialysis according to Huang, A. et al. (J. Biol. Chem. 255, 8015, 1980).

To 30 ml of dry ethyl acetate containing 3.45 mg of N-hydroxysuccinimide (NHS) 30 mmol of palmitic acid is added. The solution is kept under a nitrogen atmosphere. After further addition of 6.18 g of dicyclohexyl carbodiimide dissolved in 10 ml of dry ethyl acetate, the solution is reacted overnight at room temperature while maintaining the nitrogen atmosphere. Insoluble dicyclohexyl urea is removed by filtration using a glass fiber filter pad and vacuum, and the solvent is removed from the filtered solution by rotary evaporation under vacuum. For purification of the NHS-palmitate by recrystallization, the activated fatty acid is dissolved in a minimum quantity of hot ethanol and immediately filtered through a filter funnel containing a fluted glass fiber filter pad, both of which have been warmed to the same temperature as the ethanol solution. The NHS-palmitate is recrystallized overnight at room temperature, the solvent is removed by filtration, and the solid is dried under vacuum in a desiccator. The purity of NHS-palmitate can be analyzed by TLC on

silica plates using a mixture of chloroform: petroleum diethyl ether (bp 40-60 °C) of 8: 2 as solvent. NHS and NHS-palmitate may be detected by staining with 10% hydroxylamine in 0.1 M NaOH followed after 2 min by a 5% solution of $FeCl_3$ in 1.2 M HCl (creates red-coloured spots).

For conjugation of NHS-palmitate to antibodies, 44 μg purified NHS-palmitate dissolved in 20 mM sodium phosphate, 0.15 M NaCl, 2% deoxycholate, pH 7.4, are mixed with 2 mg antibody and incubated for 10 hrs at 37 °C. Excess palmitic acid is removed by gel filtration on Sephadex G-75 (Pharmacia) equilibrated with 20 mM sodium phosphate, 0.15 M NaCl, 0.15% deoxycholate, pH 7.4.

For incorporation of palmitate-antibody conjugates into intact liposomes, palmitate-antibody conjugates in 20 mM sodium phosphate, 0.15 M NaCl, 0.15% deoxycholate, pH 7.4, are added in a ratio of 20: 1 (w/w) to intact liposomes in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4. After the addition of deoxycholate to a final concentration of 0.7%, the suspension is mixed thoroughly using a vortex mixer, and the liposomes are dialyzed against 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4.

VI.11.4. Covalent attachment of proteinaceous affinity components derivatized with low molecular affinity components to intact liposomes

For the preparation of preformed complexes of affinity liposomes capable of binding to amplification polymers, liposomes may be prepared containing surface-attached proteinaceous affinity components (e.g., antibodies with specificity for captured antigen) which are derivatized with low molecular weight affitnity components (e.g., biotin residues for complexation via streptavidin). Covalent attachment of proteinaceous affinity components derivatized with low molecular affinity components to intact liposomes is performed as described in section VI.11.3.

VI.11.4.1. Derivatization of IgG antibodies with biotin residues

NHS-LC-biotin (succinimidyl-6-(biotinamido) hexanoate; Pierce Chemical Company, Rockford, IL, USA) is dissolved in DMF at a concentration of 40 mg/ml. An aliquot of 50 μl of this solution is added in two aliquots (apportioned 10 min apart) to each ml of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing the IgG antibody to be biotinylated at a concentration of 10 mg/ml. After 30-60 min at room temperature, unreacted biotinylation reagent and reaction-by-products are removed by gel filtration on Sephadex G-25 (Pharmacia) equilibrated with 20 mM sodium phosphate,

0.15 M NaCl, pH 7.2. The level of biotin incorporation is determined using the HABA dye (4'-hydroxyazobenzene-2-carboxylic acid) procedure (Green, N.M. Biochem. J. 94, 23c-24c, 1965).

VI.11. RELEASE OF ENCAPSULATED ENZYME ACTIVATORS FROM AFFINITY LIPOSOMES

Method A. Utilizing cholesterol-containing affinity liposomes, the release of encapsulated enzyme activators is mediated by the addition of detergent (i.e., Triton X-100 or sodium deoxycholate) or organic solvent. The percentage of detergent or organic solvent required for lysis of cholesterol-containing affinity liposomes is dependent on the percentage of cholesterol in the liposomal bilayer. For example, the integrity of the bilayer of affinity liposomes prepared from a mixture containing phosphatidyl choline, cholesterol, phosphatidyl glycerol, and phosphatidyl ethanolamine derivatized with a reactive residue at a molar ratio of 8: 10: 1: 1, will be stable up to a level of organic solvent addition of about 5%. In contrast, affinity liposomes prepared from a mixture containing only phosphatidyl choline and phosphatidyl glycerol at a molar ratio of 4: 1, are completely disrupted in the presence of 0.4% Triton X-100.

Method B. Utilizing temperature-sensitive, cholesterol-free affinity liposomes, the release of encapsulated enzyme activators is mediated by an increase of the ambient temperature (phase-transition release) as described by Magin, R.L., and Weinstein, J.N. (In: Liposome Technology (G. Gregoriadis, ed.), vol. III., pp. 137-155, CRC Press, Boca Raton, Fl., 1984). Preferred heating rates at passage through T_m are 10 to 15 °C/min.

VI.13. SYNTHESIS OF POLYMERIC CARRIER SYSTEMS FOR AMPLIFIED ASSAY PROCEDURES

Soluble dextran polymers of molecular weight between 10,000 and 50,000 have been used extensively as carriers of proteins and other molecules including the application as a carrier of biotin residues (Brandt, H.M., and Apkarian, A.V. J. Neurosci. Methods 45, 35, 1992) and hapten molecules (Shi, L.B. et al., Cancer Res. 51, 4192, 1991).

VI.13.1. Activation of dextran polymers

VI.13.1.1. Synthesis of polyaldehyde derivatives of dextran

Dextran of molecular weight between 10,000 and 40,000 is dissolved in a 30 mM aqueous sodium periodate solution (6.42 g $NaIO_4$ in 500 ml deionized water) and

stirred overnight at room temperature in the dark. Excess reactant is removed by dialyis against water and the purified polyaldehyde dextran is lyophilized for long-term storage. The degree of aldehyde formation may be assessed by aldehyde-mediated reduction of Cu^{2+} to Cu^{+} which can be detected using the bicinchoninic acid (BCA) reagent (Pierce Chemical Company, Rockford, IL, USA) as described by Smith, et al. (Anal. Biochem. 150, 76, 1985). The formation of Cu^{+} is in direct proportion to the amount of aldehydes present in the polymer. BCA forms a purple-colored complex with Cu^{+} which can be measured at 562 nm.

VI.13.1.2. Synthesis of polyamine and polyhydrazide derivatives of dextran

For the preparation of polyamine derivatives of dextran, ethylene diamine (or another suitable diamine) is dissolved in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 3 M. To avoid pH adjustment of the highly alkaline free-base form of ethylene diamine, the hydrochloride form of ethylene diamine is utilized. For the preparation of polyhydrazide derivatives of dextran, adipic acid dihydrazide (or another suitable dihydrazide compound) is dissolved in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 30 mg/ml (heating under a hot water tap is necessary to dissolve the dihydrazide compound completely at this concentration) and the pH is adjusted to 7.2 with HCl. The diamine- or dihydrazide-containing solution is used to dissolve polyaldehyde dextran (prepared as described in section VIII.12.1.1.) at a concentration of 25 mg/ml and each ml of the polyaldehyde dextran/diamine (or polyaldehyde dextran/dihydrazide) solution is mixed with 0.2 ml of 1 M sodium cyanoborohydride (in a fume hood). After reaction for at least 6 hrs at room temperature, excess diamine (or dihydrazide) and reaction-by-products are removed by dialysis.

VI.13.1.3. Synthesis of polycarboxymethyl derivatives of dextran

In a fume hood, a solution consisting of 1 M chloroacetic acid in 3 M NaOH is prepared and immediately used to dissolve dextran polymer at a concentration of 40 mg/ml. After reaction for 70 min at room temperature with stirring, the reaction is stopped by adding 4 mg/ml of solid NaH_2PO_4 and adjusting the pH to neutral with 6 M HCl. Excess reactants are removed by dialysis.

VI.13.1.4. Synthesis of lactone derivatives of polycarboxymethyl-dextran

The lactone derivative of polycarboxymethyl-dextran is prepared by refluxing polycarboxymethyl-dextran (section VIII.12.1.3.) for 5 hrs in toluene or in other anhydrous solvents as described by Heindel, N.D. et al., (Bioconjugate Chem. 5, 98, 1994). The lactone derivative is highly reactive towards amine-containing molecules.

VI.13.1.5. Synthesis of epoxy-activated derivatives of dextran

*Bis*oxirane compounds are utilized to introduce epoxide functional groups into soluble dextran polymers as described by Böcher, M., Giersch, T., and Schmid, R.D. (J. Immunol. Meth. 151, 1, 1992). Epoxide functional groups react efficiently with sulfhydryl groups at pH values ranging between 7.5 and 8.5, and with amine nucleophiles at moderate alkaline pH values (typically needing pH values of at least 9).

In a fume hood, 1,4-butanediol diglycidyl ether is mixed with an equal part of 0.6 M NaOH containing 2 mg/ml sodium borohydride. With stirring, 5 mg of dextran are added to each ml of the *bis*-epoxide solution. After reaction for 12 hrs at 25 °C, excess reactants are removed by extensive dialysis. For long-term storage, the activated dextran is lyophilized.

VI.13.1.6. Synthesis of polypyridyl disulfide derivatives of dextran

For the preparation of polypyridyl disulfide derivatives of dextran, polyamine-derivatized (or polyhydrazide-derivatized) dextran polymers (prepared as described in section VI.13.1.2.) are reacted with the heterobifunctional cross-linking reagent SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate; Pierce Chemical Company, Rockford, IL, USA).

SPDP is dissolved at a concentration of 6.2 mg/ml in DMF (makes a 20 mM stock solution) and 100 µl of this solution are added to each ml of 50 mM sodium phosphate, pH 7.5, containing the polyamine-derivatized (or polyhydrazide-derivatized) dextran polymer at a concentration of 20 mg/ml. After reaction for 3 hour at room temperature, excess reagents are removed by dialysis. The degree of pyridyl disulfide derivatization is determined by monitoring the release of the pyridine-2-thione leaving groups (characteristic absorbance at 343 nm; $\varepsilon = 8.08 \times 10^3\ M^{-1}\ cm^{-1}$) after incubation of an aliquot for 15 min at room temperature in the presence of 20 mM dithiothreitol

VI.13.1.7. Synthesis of polythioester derivatives of dextran

For the preparation of polythioester derivatives of dextran, polyamine-derivatized (or polyhydrazide-derivatized) dextran polymers (prepared as described in section VII.13.1.2.) are reacted with the heterobifunctional cross-linking reagent SATA (N-succinimidyl S-acetylthioacetate; Pierce Chemical Company, Rockford, IL, USA).

SATA is dissolved at a concentration of 8 mg/ml in DMF and 100 µl of this solution are added to each ml of 50 mM sodium phosphate, pH 7.5, containing the polyamine-derivatized (or polyhydrazide-derivatized) dextran polymer at a concentration of 20 mg/ml. After reaction for 3 hour at room temperature, excess reagents are removed by dialysis.

To deprotect the thioacetyl group, 100 µl of 500 mM hydroxylamine hydrochloride, 25 mM EDTA, pH 7.5, are added to each ml of SATA-derivatized dextran polymers and reacted for 2 hours at 37 °C. The sulfhydryl-containing dextran polymers are used immediately for further reaction with sulfhydryl-reactive molecules.

VI.13.1.8. Synthesis of polyiodoacetyl derivatives of dextran

For the preparation of polyiodoacetyl derivatives of dextran, polyamine-derivatized (or polyhydrazide-derivatized) dextran polymers (prepared as described in section VI.13.1.2.) are reacted with iodoacetic acid in the presence of the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Pierce Chemical Company, Rockford, IL, USA).

A solution of 0.1 M MES (4-morpholineethanesulfonic acid), 0.15 M NaCl, pH 4.7, containing iodoacetic acid at a concentration of 10 mM and polyamine-derivatized (or polyhydrazide-derivatized) dextran polymers at a concentration of 20 mg/ml, is added to solid EDC (10 mg per ml of polyamine-derivatized dextran/iodoacetic acid mixture), mixed well, and reacted for 3 hrs at room temperature with stirring. Excess reagents are removed by extensive dialysis.

VI.13.2. Coupling of affinity components to dextran polymers

Dextran polymers suitable for amplified assay procedures contain two types of affinity components. One of the covalently linked affinity components is capable of specifically binding to a specific captured analyte (e.g., nucleic acid-reactive affinity components such as intercalating agents or oligonucleotides). The other covalently linked affinity components are capable of specifically binding multiple affinity liposomes. Suitable affinity systems mediating the binding of affinity liposomes to dextran polymers include hapten / anti-hapten antibody affinity systems, enzyme inhibitor / enzyme affinity systems, and the biotin / (strept)avidin affinity system. For example, affinity liposomes containing surface-attached proteinaceous affinity components (e.g., anti-hapten antibodies, enzyme molecules, or (strept)avidin) may be utilized for the detection of dextran polymers containing the corresponding low molecular weight affinity partner (e.g., hapten molecules, enzyme inhibitors, or biotin

residues) and nucleic acid-reactive affinity components (e.g., intercalating agents or oligonucleotides).

VI.13.2.1. Coupling of oligonucleotides and methotrexate to polyaldehyde-dextran

In this example, hydrazide-derivatized methotrexate (a high affinity inhibitor of the enzyme dihydrofolate reductase) and hydrazide-derivatized oligonucleotides (prepared as described in section VI.7.1.) are coupled to polyaldehyde derivatives of dextran (prepared as described in section VI.13.1.1.).

VI.13.2.1.1. *Derivatization of methotrexate with a hydrazide residue*

Methotrexate (MTX)-γ-hydrazide is prepared from 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA) obtained by cleavage of MTX with carboxypeptidase G_1 (Martinelli, J.A. et al, J. Med. Chem. 22, 869, 1979), and L-glutamic acid α-*tert*-butyl γ-methyl ester with the aid of the peptide bond forming reagent diethylphosphoro cyanidate (Rosowsky, A. et al., J. Med. Chem. 24, 1450, 1981).a) Synthesis of L-glutamic acid α-*tert*-butyl γ-methyl ester. A mixture of L-glutamic acid γ-methyl ester (1.29 g, 8 mmol), t-BuOAc (60 ml), and 70% $HClO_4$ (1.26 g, 8.8 mmol) is stirred at room temperature for 3 days, cooled to 0 °C, and extracted with cold 0.5 M HCl. The acidic solution is neutralized with powdered $NaHCO_3$ and extracted with Et_2O. Washing with saturated NaCl, drying, and solvent evaporation yields the product as semisolid, which is coupled directly to APA.b) Synthesis of MTX α-*tert*-butyl γ-methyl ester. APA (2.20 g, 6 mmol), diethylphosphoro cyanidate (2.94 g, 18 mmol), and Et_3N (1.2 g, 12 mmol) are dissolved in 200 ml of dry DMF. The mixture is heated to 80 °C for 2 min and cooled back to room temperature before adding a seocnd portion of Et_3N (1.2 g, 12 mmol) and freshly prepared L-glutamic acid α-*tert*-butyl γ-methyl ester (1.30 g, 6 mmol). Heating is resumed at 80 °C for 2 hours, the solvent is removed by rotary evaporation, and the residue is taken up in $CHCl_3$. After washing with 5% $NaHCO_3$ and solvent evaporation, the product is purified by column chromatography on silica gel with 95/5 $CHCl_3$/MeOH as the eluent. c) Synthesis of MTX α-*tert*-butyl ester γ-hydrazide. Hydrazine hydrate (0.5 ml) in MeO (5 ml) is added to a solution of MTX α-*tert*-butyl γ-methyl ester (0.52 g, 1 mmol) in MeOH (15 ml), and the solution is kept at 4 °C for 3 days. After vacuum evaporation of most of the solvent, $CHCl_3$ is added with just enough MeOH to bring the solid into solution. After extraction with 5% $NaHCO_3$ and evapoaration of the organic layer, the product is purified by column chromatography on silica gel (9: 1; $CHCl_3$: MeOH). d) Synthesis of MTX γ-hydrazide. A solution of MTX α-*tert*-butyl ester γ-hydrazide (1.56 g, 3 mmol) in 1 N HCl (25 ml) is kept at 50 °C for 1 hour, then cooled, and basified to pH > 9 with 5% NaOH. After adjustment to pH ~8 with AcOH and NH_4OH, the

solution is freeze-dried. MTX γ-hydrazide is purified by chromatography on a DEAE-cellulose column which is eluted first with 0.5% NH_4HCO_3 and then with 3% NH_4HCO_3.

VI.13.2.1.2. *Derivatization of dextran-polyaldehyde with hydrazide derivatives of methotrexate and oligonucleotides*

The polyaldehyde derivative of dextran is dissolved in 0.1 M sodium bicarbonate, 0.15 M NaCl, pH 8.5, at a concentration of 20 mg/ml and mixed with a two-fold molar excess (over the molar concentration of aldehyde groups) of each MTX γ-hydrazide and 5'-hydrazide-derivatized oligonucleotides. In a fume hood, to each ml of this mixture 0.2 ml of 1 M sodium cyanoborohydride are added and the reaction mixture is incubated for at least 6 hrs at room temperature. To block remaining aldehydes, 0.2 ml of 1 M Tris-HCl, pH 8, is added to each ml of the reaction mixture, and after an additional 2 hrs at room temperature, the dextran-MTX-oligonucleotide conjugates are purified by dialysis or gel filtration using a column of Sephacryl S-200.

VI.13.2.2. Coupling of oligonucleotides and biotin to polythioester-dextran

In this example, pyridyl disulfide-derivatized oligonucleotides (prepared as described in section VI.7.3.) and pyridyl disulfide-containing biotin (biotin-HPDP; N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide; Pierce Chemical Company, Rockford, IL, USA) are coupled to deprotected polythioester-derivatives of dextran (prepared as described in section VI.13.1.7.). After deprotection of the acetylthioester groups by treatment with neutral hydroxylamine, the sulfhydryl-containing dextran polymer is used immediately for coupling of the pyridyl disulfide derivatives of biotin and the oligonucleotides. The sulfhydryl-containing dextran polymer dissolved in 50 mM sodium phosphate, 2.5 mM EDTA, 50 mM NH_2OH, pH 7.5, (20 mg sulfhydryl-containing dextran per ml) is mixed with a two-fold molar excess (over sulfhydryl groups on the dextran polymer) of both, biotin-HPDP dissolved in DMS at a concentration of 20 mM, and the pyridyl disulfide-derivatized oligonucleotides dissolved in 50 mM sodium phosphate, pH 7.5. After 3 hrs at room temperature, the dextran-biotin-oligonucleotide conjugates are purified by dialysis or gel filtration using a column of Sephacryl S-200.

VI.13.2.3. Coupling of proteins to polyaldehyde-dextran

In this example, non-derivatized IgG antibodies with specificity for captured analytes and non-derivatized streptavidin are coupled to polyaldehyde derivatives of dextran.The polyaldehyde derivative of dextran is dissolved in 0.1 M sodium bicarbonate, 0.15 M NaCl, pH 8.5, at a concentration of 20 mg/ml. To each ml of

polyaldehyde-dextran solution 5 mg IgG antibody dissolved in 0.5 ml of 0.1 M sodium bicarbonate, 0.15 M NaCl, pH 8.5, and 5 mg streptavidin dissolved in 0.5 ml of 0.1 M sodium bicarbonate, 0.15 M NaCl, pH 8.5, are added. In a fume hood, to each ml of this mixture 0.2 ml of 1 M sodium cyanoborohydride is added and the reaction mixture is incubated for at least 6 hrs at room temperature. To block remaining aldehydes, 0.2 ml of 1 M Tris-HCl, pH 8, is added to each ml of the reaction mixture, and after an additional 2 hrs at room temperature, the dextran-antibody-streptavidin conjugates are purified by gel filtration using a column of Sephacryl S-200 or S-300.

VI.14. BASIC ASSAY PROCEDURE

Detection of mouse IgG using enzyme activator-containing affinity liposomes

VI.14.1. Assay principle

Mouse IgG is used as proteinaceous antigen to demonstrate the detection sensitivity of the basic assay procedure. Rat anti-mouse IgG covalently immobilized onto Sepharose beads serves as capture antibody. Captured mouse IgG is detected with rat anti-mouse IgG conjugated to streptavidin, followed by binding of biotin-labeled affinity liposomes containing $ZnCl_2$. Bound liposomes are lysed by the addition of detergent and released $ZnSO_4$ is used to activate zinc-free alkaline phosphatase covalently immobilized onto the electrode surface. p-Aminophenol (PAP) generated from p-aminophenylphosphate (PAPP) by activated alkaline phosphatase is quantified via redox recycling using interdigitated array (IDA) electrodes. The assay is performed as a combination of liquid chromatography with electrochemical detection (LCEC).

VI.14.2. Instrumentation

The analytical system applied for the detection of mouse IgG includes a micromachined flow-through assembly, a multipotentiostat, pumps, valves, and a computer (PC type). The flow is accomplished using a peristaltic pump combined with a 6-way selector valve. The micromachined flow-through assembly consists of three main parts: an immunoreaction chamber, a flow chamber designed as a channel structure, and the interdigitated electrode array (IDA). The immunoreaction chamber (volume: 100 µl) contains Sepharose beads with covalently immobilized rat

anti-mouse IgG and is connected via a two-way selector valve to a flow channel fabricated by double-sided anisotropic etching in silicon wafers. The lateral dimensions of the flow channel corresponds to the active electrode area (1mm x 3mm) and the channel hight is set to 200 µm. The inlet and outlet tubes are pasted in an acrylic holder and arranged on top of the electrode. The microelectrode arrays consist of gold electrodes fabricated on thermal oxidized silicon wafers by photolithography and the lift-off technique.One chip contains four independent interdigitated electrode pairs and has an area of 8 mm x 8 mm. One of the 70 fingers of each electrode is 1.5 µm wide. Two adjacent fingers are 0.8 µm spaced. The surface of the gold electrodes carries covalently immobilized zinc-free, inactive alkaline phosphatase.

VI.14.3. Assay components

VI.14.3.1. Immobilized capture antibodies. Rat anti-mouse IgG is immobilized onto tresyl-activated Sepharose beads as described in example VI.4.2. (Meth. Enzymol. 104, 56, 1984).

VI.14.3.2. Immobilized zink-free, inactive alkaline phosphatase. Zink-free alkaline phosphatase is prepared as described in example VI.1.1.1. (Biochemistry 13, 3754, 1974). For immobilization of the inactive enzyme molecules onto the surface of the gold electrodes, the electrodes are derivatized with a heterobifunctional reagent containing a terminal mercapto residue and a carboxy group. The carboxy group is used to couple zink-free alkaline phosphatase in a carbodiimide-mediated reaction.

VI.14.3.3. Biotinylated affinity liposomes containing $ZnSO_4$. Biotinylated affinity liposomes are prepared by the injection method (Biochim. Biophys. Acta 298, 1015, 1973) from a lipid mixture of dimyristoylphosphatidylcholine, cholesterol, dicetylphosphate at a molar ratio of 5:4:1, and N-biotinyldipalmitoyl-L-α-phosphatidylethanolamine (B-PE; Molecular Probes, Junction City, OR, USA) at a concentration of 0.1mol% of total lipid. To prepare liposomes, 2 µmol of stock lipid mixture in chloroform is evaporated under a stream of nitrogen and then placed in a vacuum desiccator overnight. The lipid is resolubilized in 50 µl of dry isopropanol and

injected with a syringe into 1 ml of a 2 M $ZnSO_4$ solution which is being mixed by vortex. Liposomes of uniform size are formed spontaneously by this method. Unencapsulated $ZnSO_4$ is removed by gel filtration.

VI.14.3.4. p-Aminophenylphosphate (PAPP). PAPP is synthesized from p-nitrophenylphosphate as described (Biochemistry, 20, 1606, 1981).

VI.14.4. Assay procedure

First, the analytical system is washed with several volumes of 50 mM HEPES, 1 mM EDTA, pH 7.2. Thereafter, the valve connecting the immunoreaction chamber with the flow chamber is closed and the sample (50 µl) containing mouse IgG is applied to the immunoreaction chamber. After an incubation of 10 min at room temperature, the immunoreaction chamber is washed with several volumes of 50 mM HEPES, pH 7, and captured mouse IgG is detected by the addition of 50 µl rat anti-mouse IgG-streptavidin conjugate in 50 mM HEPES, pH 7. After an incubation for 5 min at room temperature, the immunoreaction chamber is washed again with several volumes of 50 mM HEPES, pH 7, followed by the addition of 50 µl biotinylated affinity liposomes containing $ZnSO_4$. After an incubation of 5 min at room temperature, the immunoreaction chamber is washed with several volumes of 20 mM metal-free sodium carbonate buffer, pH 9.5. Finally, the valve connecting the immunoreaction chamber with the flow chamber is opened and bound affinity liposomes are lysed by the addition of 100 µl of 20 mM metal-free sodium carbonate buffer, pH 9.5, containing 0.01% Triton X-100 and 5 mM p-aminophenylphosphate (PAPP). Activated alkaline phosphatase is detected by redox recycling of generated p-aminophenol (PAP). The PAP enzymatically generated from PAPP is oxidized to quinoneimine at the anode (+250 mV) yielding an oxidation current. At the cathode (-50 mV) the quinoneimine is reduced to PAP.

VI.14.5. Detection sensitivity

Using redox recyling for the detection of PAP, the detection limit is in the range of 50 nM corresponding to 2.5 pmol per 50 µl. Considering the high turn over number of alkaline phosphatase (approximately 3000 s^{-1}), an incubation period of 120 s allows

the detection of alkaline phosphatase in the range of 10 attomol (10^{-17} mol). Since the release of approximately 10^5 Zn^{2+} ions from a single bound affinity liposome reactivates many immobilized alkaline phosphatese molecules (1 mm^2 of electrode surface area accommodates approximately 4 x 10^{10} alkaline phosphatase molecules from *E. coli* based on a molecular weight of approximately 89 kD and an immobilization area of 25 nm^2 per enzyme molecule), the described assay configuration allows the detection of mouse IgG in the zeptomol range (<100 fg/50 µl).

VI.15. AMPLIFIED ASSAY PROCEDURE

Detection of mouse IgG using preformed complexes of enzyme activator-containing affinity liposomes

VI.15.1. Assay principle

Mouse IgG is used as proteinaceous antigen to demonstrate the detection sensitivity of the basic assay procedure. Rat anti-mouse IgG covalently immobilized onto Sepharose beads serves as capture antibody. Captured mouse IgG is detected with biotinylated rat anti-mouse IgG bound to preformed complexes of avidin and biotin-labeled affinity liposomes containing $ZnCl_2$. Bound complexes of biotinylated anti-mouse IgG, avidin, and biotinylated liposomes are lysed by the addition of detergent and released $ZnSO_4$ is used to activate zinc-free alkaline phosphatase covalently immobilized onto the electrode surface. p-Aminophenol (PAP) generated from p-aminophenylphosphate (PAPP) by activated alkaline phosphatase is quantified via redox recycling using interdigitated array (IDA) electrodes. The assay is performed as a combination of liquid chromatography with electrochemical detection (LCEC).

VI.15.2. Instrumentation

The analytical system applied for the detection of mouse IgG using the amplified assay procedure is the same as described in VI.14.2.

VI.15.3. Assay components

VI.15.3.1. Immobilized capture antibodies. Rat anti-mouse IgG is immobilized onto tresyl-activated Sepharose beads as described in example VI.4.2. (Meth. Enzymol. 104, 56, 1984).

VI.15.3.2. Immobilized zink-free, inactive alkaline phosphatase. Zink-free alkaline phosphatase is prepared and immobilized onto the surface of the gold electrodes as described in VI.14.3.2.

VI.15.3.3. Preformed complexes of biotinylated anti-mouse IgG, avidin, and biotinylated affinity liposomes containing $ZnSO_4$. Biotinylated affinity liposomes containing $ZnSO_4$ are prepared as described in VIII.14.3. After removal of unencapsulated $ZnSO_4$ by gel filtration, liposomes are continuously stirred in 20 mM Tris, 0.15 M NaCl, 0.01 % (v/v) NaN_3, pH 7.4, at a concentration of 0.5 nmol total lipid in 2.5 ml buffer. Rat anti-mouse IgG is derivatized with a 100-fold molar excess of succinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin) to IgG according to standard protocols and passed over a Sephadex G-50 column before use. Avidin is quantified using an extinction coefficient of 15.4 cm^{-1} for a 1% (w/v) solution at 281 nm. Preformed complexes of biotinylated anti-mouse IgG (B-Ab), avidin, and biotinylated affinity liposomes are prepared by mixing biotinylated affinity liposomes containing 2 nmol of N-biotinyldipalmitoyl-L-α-phosphatidylethanolamine (B-PE) at a level of 0.1 mol% of total lipid with 0.4 nmol of avidin (B-PE:avidin ratio = 5) for 2 min, followed by 1.2 nmol of biotinylated anti-mouse IgG (B-Ab:avidin ratio = 3) for 10 min. Using these ratios, the affinity liposomes are protected from aggregation and maintain their original size (approximately 100 nm).

VI.15.3.4. p-Aminophenylphosphate (PAPP). PAPP is synthesized from p-nitrophenylphosphate as described (Biochemistry, 20, 1606, 1981).

VI.15.4. Assay procedure

First, the analytical system is washed with several volumes of 50 mM HEPES, 1 mM EDTA, pH 7.2. Therafter, the valve connecting the immunoreaction chamber with the

flow chamber is closed and the sample (50 µl) containing mouse IgG is applied to the immunoreaction chamber. After an incubation of 10 min at room temperature, the immunoreaction chamber is washed with several volumes of 50 mM HEPES, pH 7, and captured mouse IgG is detected by the addition of 50 µl preformed complexes of biotinylated anti-mouse IgG, avidin, and biotinylated affinity liposomes containing $ZnSO_4$ in 20 mM Tris, 0.15 M NaCl, 0.01 % (v/v) NaN_3, pH 7.4. After an incubation for 5 min at room temperature, the immunoreaction chamber is washed with several volumes of 20 mM metal-free sodium carbonate buffer, pH 9.5. Finally, the valve connecting the immunoreaction chamber with the flow chamber is opened and bound complexes of affinity liposomes are lysed by the addition of 100 µl of 20 mM metal-free sodium carbonate buffer, pH 9.5, containing 0.01% Triton X-100 and 5 mM p-aminophenylphosphate (PAPP). Activated alkaline phosphatase is detected by redox recycling of generated p-aminophenol (PAP). The PAP enzymatically generated from PAPP is oxidized to quinoneimine at the anode (+250 mV) yielding an oxidation current. At the cathode (-50 mV) the quinoneimine is reduced to PAP.

VI.15.5. Detection sensitivity

As compared to the detection sensitivity of the basic assay procedure (VI.14.5), the use of preformed complexes of biotinylated anti-mouse IgG, avidin, and biotinylated affinity liposomes increases the sensitivity by approximately one order of magnitude.

* * *

Claims:

1. A reporter system for detecting an analyte present in a liquid, comprising the following components:

a) capture molecules immobilized on a solid support and capable of specifically binding said analyte;

b) enzyme-activator containing affinity components, selected from

b1) affinity liposomes containing encapsulated enzyme activator and comprising at least one surface-attached affinity component capable of specifically binding to said analyte and/or said capture molecules in a condition where analyte and capture molecules are bound to each other, but not to free capture molecules,

b2) (i) polymeric carrier molecules containing at least one covalently linked affinity component capable of specifically binding to to analyte and/or capture molecules in a condition where analyte and the capture molecules are bound to each other, but not to free capture molecules, and at least two covalently linked affinity components capable of specifically binding to affinity liposomes (ii), plus (ii) said affinity liposomes containing encapsulated enzyme activator and comprising at least one surface-attached affinity component capable of specifically binding to said polymeric carrier molecules;

b3) complexes of affinity liposomes, the liposomes containing encapsulated enzyme activator and comprising at least one surface-attached affinity component capable of specifically binding to said analyte and/or said capture molecules in a condition where analyte and capture molecules are bound to each other, but not to free capture molecules; or

b4) (i) polymeric carrier molecules containing at least one covalently linked affinity component capable of specifically binding to said analyte and/or said capture molecules in a condition where analyte and capture molecules are bound to each other, but not to free capture molecules, and more than one

covalently linked affinity component capable of specifically binding to affinity liposomes of the complexes (ii), plus (ii) said complexes of affinity liposomes containing encapsulated enzyme activator and comprising at least one surface-attached affinity component capable of specifically binding to said polymeric carrier molecules;

c) inactive enzyme molecules immobilized on a solid support and capable of being activated by the said enzyme activator encapsulated in the said affinity liposomes, and

d) a substrate not detectable by the said reporter system which in the presence of activated enzyme molecules (c) is convertible into reporter molecules capable of being detected by the reporter system.

2. The reporter system according to claim 1, wherein the reporter molecules are optical detectable molecules and the reporter system is an optical reporter system.

3. The reporter system according to claim 1, wherein the reporter molecules are electrochemically detectable molecules, the reporter system further comprising:
   e) an electrochemical sensor comprising a closely spaced array of thin film noble metal electrodes for voltammetric detection of said reporter system.

4. The reporter system according to claim 1, wherein the capture molecules and the inactive enzyme molecules are immobilized on different supports.

5. The reporter system according to claim 4, wherein the capture molecules are immobilized on a substantially flat support, preferably on a structure of a container in which detection or part thereof is intended to take place, and the inactive enzyme molecules are immobilized on a support which may be introduced into the said container, preferably having the shape of a strip or of beads, or wherein the inactive enzyme molecules are immobilized on a substantially flat support, preferably on a structure of a container in which detection or part thereof is intended to take place, and the capture molecules are immobilized on a support which may be introduced into the said container, preferably having the shape of a strip or beads.

6. The reporter system of claim 1, wherein the or at least one surface-attached affinity component(s) of the affinity liposomes is capable of specifically binding to captured analyte being selected from the group consisting of nucleic acid-reactive components, antigens including haptens, immunoglobulins and fragments of immunoglobulins, non-immunoglobulin binding proteins, peptides, and non-biologic binding molecules and binding to the said captured analyte in a structure-restricted manner.

7. The reporter system of claim 6, wherein the or at least one of the surface-attached affinity component(s) of the affinity liposomes is a nucleic acid-reactive component selected from the group of single-stranded ribo and deoxyribo nucleic acids, single-stranded ribo and deoxyribo oligonucleotides, 'preorganized' oligonucleotide structures including peptide nucleic acid (PNA) analogs, intercalating agents, intercalating agents conjugated to single-stranded oligonucleotides or nucleic acids, immunoglobulins or fragments of immunoglobulins with specificity for double- and/or triple-stranded nucleic acids, and non-immunoglobulin proteins capable of specifically binding to double-stranded nucleic acids.

8. The reporter system of claim 6, wherein the or at least one of the surface-attached affinity component(s) of the affinity liposomes is attached to the surface of the said affinity liposomes via a spacer molecule, preferably selected from oligoethylene glycol derivatives and monomers to oligomers of aminoalkyl acid derivatives.

9. The reporter system of claim 1, wherein the inactive enzyme molecules are selected from the group consisting of apometalloenzymes and non-metalloenzymes both of which are only catalytically active in the presence of certain metals, inactive deletion mutant enzymes which require complementation by certain polypeptides to be catalytically active, and incomplete, inactive enzyme complexes which require the presence of additional enzyme subunits to be catalytically active.

10. The reporter system of claim 1, the substrate being selected from the group consisting of phosphomonoester derivatives of optical reporter molecules or redox mediators for metallophosphatases, phosphodiester derivatives of optical reporter molecules or redox mediators for venom exonuclease, amino acid derivatives of optical reporter molecules or redox mediators for metalloaminopeptidases and metallocarboxypeptidases, glycoside derivatives of redox mediators for glycosidases including galactoside derivatives of redox mediators for β-galactosidase, galactoside derivatives of optical reporter molecules for β-galactosidase, and NAD^+ as a cofactor of metallodehydrogenases.

11. The reporter system of claim 10, the derivatives of redox mediators being selected from a group including p-aminophenol and derivatives thereof, catechol and derivatives thereof, dopamine and derivatives thereof, methoxytyraminin and derivatives thereof, aromatic compounds with more than one aromatic ring structure including naphthol and anthracene derivatives, and heterocyclic aromatic compounds including serotonin, hydroxyindol acetic acid, and derivatives thereof.

12. The reporter system of claim 3, wherein the electrochemical sensor is a microelectronic interdigitated array of electrodes where anodes and cathodes have a width between 100 and about 800 nm and the electrodes are spaced apart from each other with a distance between 5 and 5000 nm.

13. The reporter system according to claim 1, wherein the polymeric carrier molecules are selected from the group consisting of derivatives of synthetic or natural polymers including polysaccharides, poly(amino acids), poly(vinyl alcohols), poly(vinylpyrrolidinones), poly(acrylic acids), polyurethanes, polyphosphazenes, and copolymers of such polymers.

14. The reporter system of claim 13, wherein the covalently linked affinity component of the said polymeric carrier molecules capable of specifically binding to the said affinity liposomes is selected from the group consisting of haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin.

15. The reporter system of claim 1, wherein the surface-attached affinity components of the said affinity liposomes are selected from the group consisting of haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin.

16. The reporter system of claim 1, wherein the complexes of affinity liposomes b3) are formed from affinity liposomes containing two types of surface-attached affinity components, the type I surface-attached affinity components being capable of specifically binding to said analyte and/or said capture molecules in a condition where analyte and capture molecules are bound to each other, but not to free capture molecules, and preferably defined as in claim 6, and the type II surface-attached affinity components being capable of mediating the complexation of affinity liposomes via bridging molecules which have at least two binding sites for type II affinity components.

17. The reporter system of claim 16, wherein the surface-attached type II affinity components capable of mediating the complexation of affinity liposomes via bridging molecules are selected from a group including haptens, enzyme inhibitors, and biotin.

18. The reporter system of claim 16, wherein the bridging molecules are selected from molecules with more than one binding site, preferably from the group consisting of a) bi- or oligovalent anti-hapten antibodies, fragments thereof, conjugates thereof, and fusion constructs thereof; b) inhibitor-binding enzymes, conjugates thereof, and fusion constructs thereof; and c) avidin and streptavidin.

19. The reporter system of claim 1, wherein the complexes of affinity liposomes b3) are formed from affinity liposomes containing two types of surface-attached affinity components, the type I surface-attached affinity components being capable of specifically binding to captured analytes in a condition where analyte and capture molecules are bound to each other, but not to free capture molecules, and the type II surface-attached affinity components being capable of mediating the complexation of affinity liposomes via polymeric carrier molecules with immobilized affinity components providing at least two binding sites for type II affinity components .

20. The reporter system of claim 19, wherein the surface-attached type II affinity components capable of mediating the complexation of affinity liposomes via polymeric carrier molecules are selected from haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin.

21. The reporter system of claim 19, wherein the polymeric carrier molecules are selected from the group consisting of derivatives of synthetic or natural polymers including polysaccharides, poly(amino acids), poly(vinyl alcohols), poly(vinylpyrrolidinones), poly(acrylic acids), various polyurethanes, polyphosphazenes, and copolymers of such polymers.

22. The reporter system of claim 19, wherein the affinity components immobilized on the said polymeric carrier molecules are selected from the group consisting of haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin.

23. The reporter system of claim 1, wherein the complexes of affinity liposomes b3) are formed from two types of affinity liposomes, type I affinity liposomes containing at least two surface-attached affinity components capable of specifically binding to captured analytes, preferably being defined as in claim 6, and type II affinity liposomes containing at least two surface-attached affinity components capable of specifically binding to the surface-attached affinity components of type I affinity liposomes.

24. The reporter system of claim 23, wherein the affinity components of type I affinity liposomes are selected from oligonucleotides capable of forming specific helical complexes with specific target nucleic acids or their amplicons, and the affinity components of type II affinity liposomes are selected from oligonucleotides capable of forming specific helical complexes with the oligonucleotides of type I affinity liposomes.

25. The reporter system of claim 23, wherein the affinity components of type I affinity liposomes are selected from molecules capable of specifically binding to captured analytes including immunoglobulins or fragments thereof, non-immunoglobulin binding proteins, and peptides, and the affinity components of type II affinity liposomes are selected from immunoglobulins or fragments thereof with specificity for the affinity components of type I affinity liposomes.

26. The reporter system of claim 1, wherein the complexes of affinity liposomes b4) (ii) are formed from affinity liposomes containing two types of surface-attached affinity components, the surface-attached type I affinity components being capable of specifically binding to polymeric carrier molecules and preferably being selected from the group consisting of haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin, and the surface-attached type II affinity components being capable of mediating the complexation of affinity liposomes via bridging molecules and preferably being selected from the group consisting of haptens, enzyme inhibitors, and biotin, the bridging molecules being selected from molecules with more than one binding site, preferably from the group consisting of a) bi- or oligovalent anti-hapten antibodies, fragments thereof, conjugates thereof, and fusion constructs thereof; b) inhibitor-binding enzymes, conjugates thereof, and fusion constructs thereof; and c) avidin and streptavidin and providing at least two binding sites for type II affinity components.

27. The reporter system of claim 1, wherein the complexes of affinity liposomes b4) (ii) are formed from affinity liposomes containing two types of surface-attached affinity components, the surface-attached type I affinity components being capable of specifically binding to polymeric carrier molecules and preferably being selected from the group consisting of haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin, and the surface-attached type II affinity components being capable of mediating complexation of affinity liposomes via polymeric carrier molecules having immobilized affinity components selected from the group consisting of haptens, antihapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin, and streptavidin, the bridging molecules being selected from molecules with more than one binding site including a) bi- or oligovalent anti-hapten antibodies, fragments thereof, conjugates thereof, and

fusion constructs thereof; b) inhibitor-binding enzymes, conjugates thereof, and fusion constructs thereof; and c) avidin and streptavidin and providing at least two binding sites for type II affinity components.

28. A method for detecting an analyte in a liquid sample, comprising the steps of:

a) providing
(i) capture molecules immobilized on a solid support and capable of specifically binding said analyte;
(ii) affinity liposomes containing encapsulated enzyme activator, the affinity liposomes or components bound or later to be bound thereto specifically binding to said analyte and/or said capture molecules in a condition where analyte and capture molecules are bound to each other, but not to free capture molecules,
(iii) enzyme molecules in an inactive condition immobilized on a solid support,
(iv) a substrate being convertible to reporter molecules via said enzyme molecules in activated condition,

b) contacting the sample with the immobilized capture molecules;

c) adding said enzyme activator-containing affinity liposomes;

d) removing unbound affinity liposomes;

e) releasing encapsulated enzyme activator from the interior of affinity liposomes bound to analyte and/or capture molecules as defined above,

f) bringing the immobilized, inactive enzyme molecules into contact with enzyme activator released from the interior of said affinity liposomes and restoring the activity of said enzyme molecules;

g) adding the substrate being convertible into reporter molecules to the said enzyme molecules,
and
h) measuring the reporter molecules formed by activted enzyme molecules.

29. The method according to claim 28, wherein the enzyme activator is released from the interior of the liposomes by increasing the ambient temperature or by adding a liposome-lysing agent.

30. The method according to claim 28, wherein the analyte to be detected is selected from antigens, antibodies, nucleic acids, or amplicons thereof.

31. The method according to claim 28, wherein the analyte to be detected is selected from nucleic acid sequences and amplicons thereof, a nd wherein the capture molecules are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to that of the analyte to be detected or a selected part thereof and the affinity liposomes are selected from the group of affinity liposomes having surface-attached intercalating agents or surface-attached antibodies, both being capable of specifically binding to double-stranded nucleic acids.

32. The method according to claim 28, wherein the analyte to be detected is selected from nucleic acid sequences and amplicons thereof, and wherein the capture molecules are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to a first segment of the sequence of the analyte to be detected and the affinity liposomes are selected from the group of affinity liposomes having surface-attached single-stranded oligonucleotides comprising a sequence complementary to a second segment of the sequence of the analyte to be detected or having surface-attached single-stranded oligonucleotide-intercalating agent conjugates being capable of specifically binding to double-stranded nucleic acids.

33. The method according to claim 28, additionally comprising the steps of

(i) adding polymeric carrier molecules capable of specifically binding to said analyte and/or said capture molecules in a condition where analyte and capture molecules are bound to each other, but not to free capture molecules, the polymeric carrier moleules being the said components bound or later to be bound to the said affinity liposomes by providing more than one binding site for said affinity liposomes per each of the said

polymeric carrier molecules, and

(j) removing unbound polymeric carrier molecules,

before step (c) is performed, and wherein the affinity liposomes added in step (c) are capable of specifically binding to the said analyte-bound polymeric carrier molecules.

34. The method according to claim 33, wherein the analyte to be detected is selected from nucleic acid sequences and amplicons thereof, wherein the capture molecules are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to that of the analyte to be detected or a selected part thereof and the polymeric carrier molecules added according to step (i) are capable of specifically binding to captured analyte via covalently linked intercalating agents or covalently linked antibodies both being capable of specifially binding to double-stranded nucleic acids.

35. The method according to claim 33, wherein the analyte to be detected is selected from nucleic acid sequences and amplicons thereof, and wherein the capture molecules are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to a first segment of the sequence of the analyte to be detected and the polymeric carrier molecules added according to step (i) are capable of specifically binding to captured analyte via covalently linked single-stranded oligonucleotides or covalently linked single-stranded oligonucleotide-intercalating conjugates, said oligonucleotides having a sequence complementary to a second segment of the nucleic acid sequence of the analyte to be detected, and said conjugated intercalating agents being capable of specifically binding to double stranded nucleic acids, respectively.

36. The method according to claim 28, wherein the enzyme activator-containing affinity liposomes added in step (c) are preformed complexes of said affinity complexes.

37. The method according to claim 36, wherein the analyte to be detected is selected from nucleic acid sequences and amplicons thereof, and wherein the capture molecules are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to that of the analyte to be detected or a selected part thereof, and the preformed complexes of affinity liposomes are selected from the group of complexes containing affinity liposomes having surface-attached intercalating agents or surface-attached antibodies, both being capable of specifically binding to double-stranded nucleic acids.

38. Method according to claim 36, wherein the analyte to be detected is selected from nucleic acid sequences and amplicons thereof, and wherein the capture molecules are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to a first segment of the sequence of the analyte to be detected and the preformed complexes of affinity liposomes are selected from the group of complexes containing affinity liposomes having surface-attached single-stranded oligonucleotides comprising a sequence complementary to a second segment of the sequence of the analyte to be detected or having surface-attached single-stranded oligonucleotide-intercalating agent conjugates being capable of specifically binding to double-stranded nucleic acids.

39. Method according to claim 28, comprising the steps of

(i) adding polymeric carrier molecules capable of specifically binding to said analyte and/or said capture molecules in a condition where analyte and capture molecules are bound to each other, but not to free capture molecules, the polymeric carrier moleules being the said components bound or later to be bound to the said affinity liposomes by providing more than one binding site for said affinity liposomes per each of the said polymeric carrier molecules, and

(j) removing unbound polymeric carrier molecules,

before step (c) is performed, and wherein the affinity liposomes added in step (c) are preformed complexes of said affinity complexes capable of specifically binding to the said analyte-bound polymeric carrier molecules.

40. Method according to claim 39, wherein the analyte to be detected is selected from nucleic acid sequences and amplicons thereof, and wherein the capture molecules are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to that of the analyte to be detected or a selected part thereof, and the polymeric carrier molecules added according to step (i) are capable of specifically binding to captured analyte via covalently linked intercalating agents or covalently linked antibodies both being capable of specifially binding to double-stranded nucleic acids.

41. Method according to claim 39, wherein the analyte to be detected is selected from nucleic acid sequences and amplicons thereof, and wherein the capture molecules are selected from single-stranded nucleic acids or oligonucleotides with a sequence complementary to a first segment of the sequence of the analyte to be detected and the polymeric carrier molecules added according to step (i) are capable of specifically binding to captured analyte via covalently linked single-stranded oligonucleotides or covalently linked single-stranded oligonucleotide-intercalating conjugates, said oligonucleotides having a sequence complementary to a second segment of the nucleic acid sequence of the analyte to be detected, and said conjugated intercalating agents being capable of specifically binding to double stranded nucleic acids, respectively.

42. A method for detecting an analyte in a liquid sample,

a) providing
(i) capture molecules immobilized on a solid support and capable of specifically binding said analyte;
(ii) affinity liposomes containing encapsulated enzyme activator and having the analyte to be analyzed attached to their surface,
(iii) enzyme molecules in an inactive condition immobilized on a solid support,
(iv) a substrate being convertible to reporter molecules via said enzyme molecules in activated condition,

b) mixing the sample with a limited quantity of the said affinity liposomes,

c) contacting the mixture obtained in (b) with the immobilized capture molecules,

d) removing unbound affinity liposomes;

e) releasing encapsulated enzyme activator from the interior of affinity liposomes bound to capture molecules,

f) bringing the immobilized, inactive enzyme molecules into contact with enzyme activator released from the interior of said affinity liposomes and restoring the activity of said enzyme molecules;

g) adding the substrate being convertible into reporter molecules to the said enzyme molecules,
and

h) measuring the reporter molecules formed by activated enzyme molecules.

43. The method according to claim 42, wherein the analyte is a low molecular weight analyte.

44. The method according to claim 42, wherein the enzyme activator is released from the interior of the liposomes by increasing the ambient temperature or by adding a liposome-lysing agent.

45. A method for detecting an analyte in a liquid sample,

a) providing
(i) capture molecules immobilized on a solid support and capable of specifically binding said analyte;
(ii) analyte molecules labeled with an affinity component capable of binding affinity liposomes containing surface-attached complementary affinity components,
(iii) affinity liposomes containing encapsulated enzyme activator and having the said complementary affinity components attached to their surface,

(iv) enzyme molecules in an inactive condition immobilized on a solid support,
(v) a substrate being convertible to reporter molecules via said enzyme molecules in activated condition,

b) mixing the sample with a limited quantity of analyte molecules mentioned in (ii)

c) contacting the mixture obtained in (b) with the immobilized capture molecules,

d) adding affinity liposomes according to (iii)

e) removing unbound affinity liposomes;

f) releasing encapsulated enzyme activator from the interior of affinity liposomes bound to labeled analyte,

g) bringing the immobilized, inactive enzyme molecules into contact with enzyme activator released from the interior of said affinity liposomes and restoring the activity of said enzyme molecules;

h) adding the substrate being convertible into reporter molecules to the said enzyme molecules,
and
h) measuring the reporter molecules formed by activated enzyme molecules.

* * *

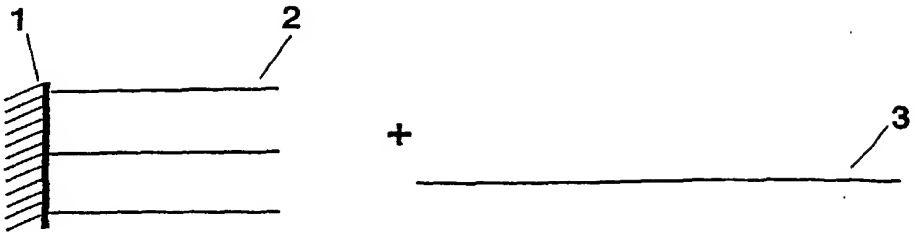

Fig.1a

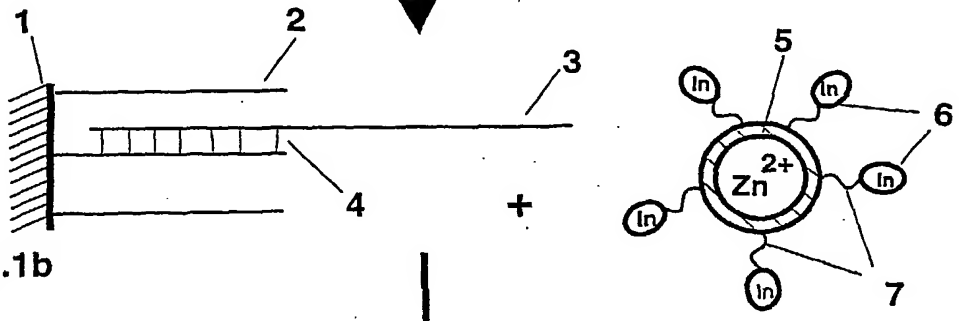

Fig.1b

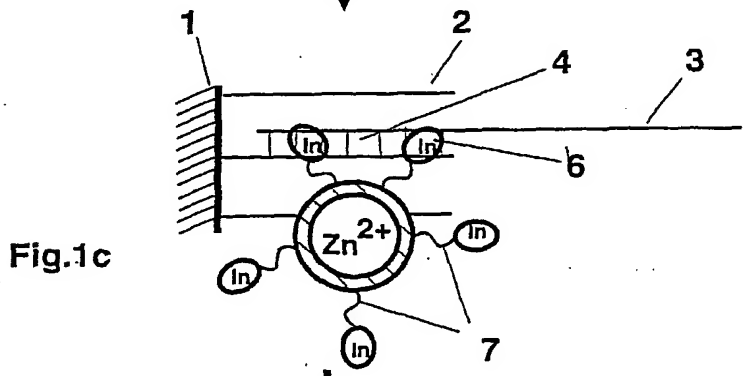

Fig.1c

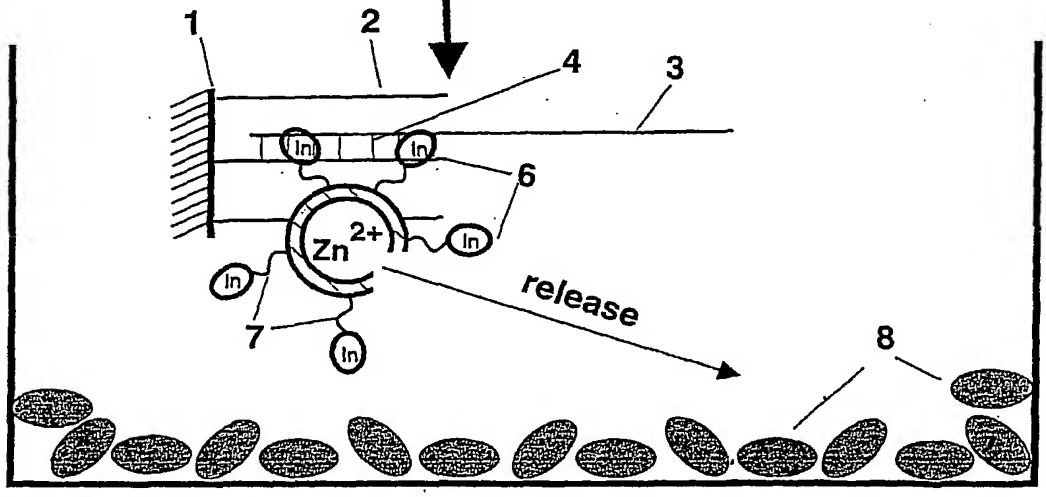

Fig1.d

Fig.1

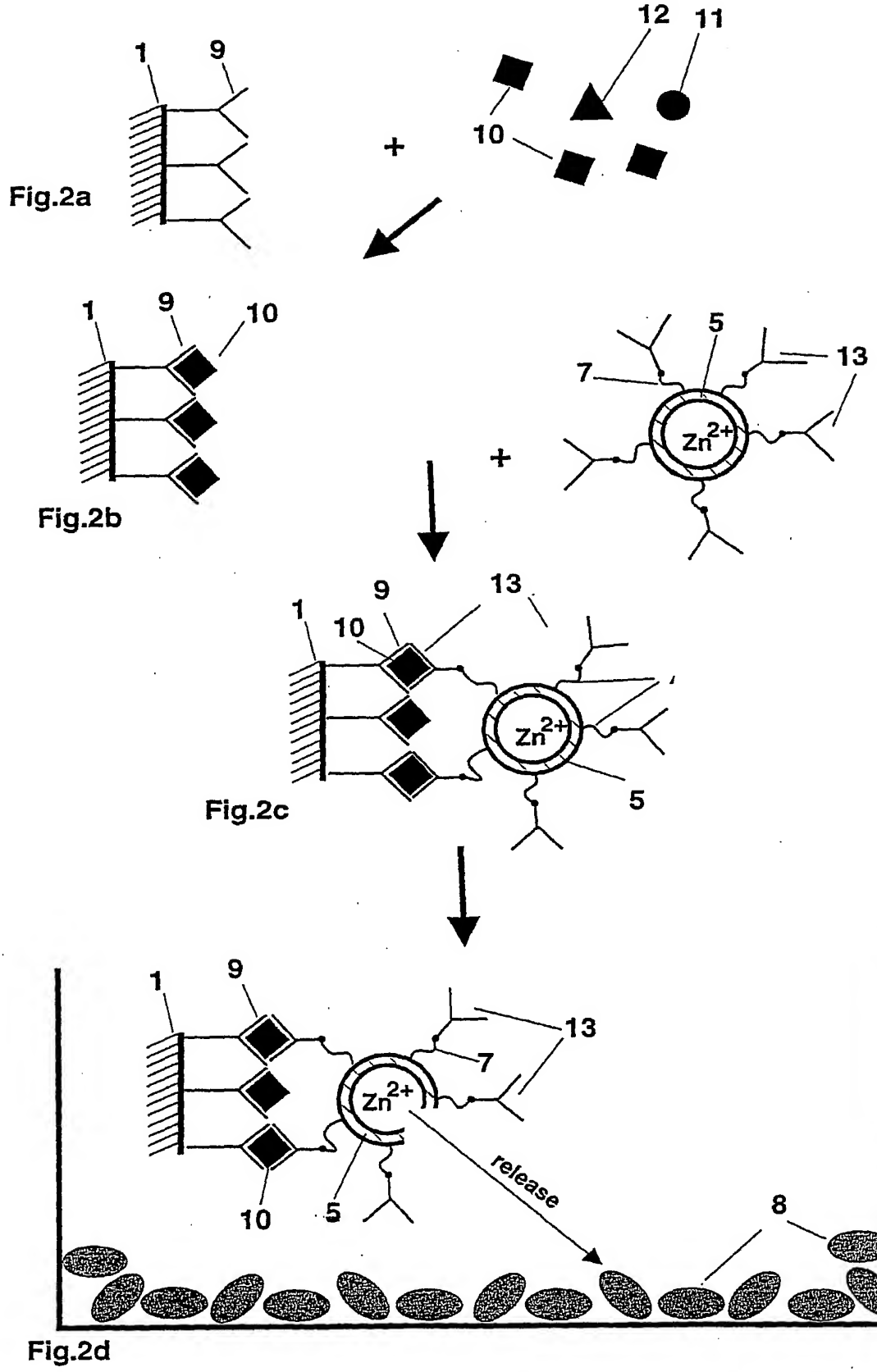
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Fig.2a
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Fig.2d

Fig.2

DERWENT-ACC-NO: 2003-120408

DERWENT-WEEK: 200456

TITLE: Reporter system for detecting analyte in liquid, comprises solid supports containing immobilized capture molecules, and immobilized inactive enzyme molecules, enzyme activator-containing affinity liposomes, and substrates

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ABSTRACTED-PUB-NO: WO 02082078 A2

BASIC-ABSTRACT:

NOVELTY - A reporter system (I) for detecting an analyte (A) present in a liquid, comprises a solid support (1) containing immobilized capture molecules (2), and a solid support containing immobilized inactive enzyme molecules, enzyme activator-containing affinity liposomes, and substrates for detection procedures.

DESCRIPTION - A reporter system (I) for detecting (A) present in a liquid, comprises the following components:

(i) capture molecules (C) immobilized on a solid support (SS) and capable of specifically binding (A);

(ii) enzyme-activator containing affinity components, chosen from:

(i) affinity liposomes (II) containing encapsulated enzyme activator and comprising a surface-attached affinity component capable of specifically binding to (A) and/or the (C) in a condition, where (A) and (C) are bound to each other, but not to free (C);

(ii) polymeric carrier molecules containing a covalently linked affinity component capable of specifically binding to (A) and/or (C) in a condition where (A) and (C) are bound to each other, but not to free (C), and two covalently linked affinity components capable of specifically binding to affinity liposomes, plus affinity liposomes (III) which contain encapsulated enzyme activator and comprising a surface-attached affinity component capable of specifically binding to the polymeric carrier molecules;

(iii) complexes of (II); or

(iv) polymeric carrier molecules containing a covalently linked affinity component capable of specifically binding to (A) and/or (C) in a condition where (A) and (C) are bound to each other, but not to free (C), and more than one covalently linked affinity components capable of specifically binding to affinity liposomes, plus the complexes of (III);

(iii) inactive enzyme molecules immobilized on a SS and capable of being activated by the enzyme

activator encapsulated in the affinity liposomes; and

(iv) a substrate not detectable by the reporter system which in the presence of activated enzyme molecules is convertible into reporter molecules capable of being detected by the reporter system.

USE - (I) is useful for detecting an analyte (antigens, antibodies, nucleic acids or amplicons) in a liquid sample, which involves:

(a) providing (I);

(b) contacting the sample with the immobilized (C);

(c) adding the enzyme activator-containing affinity liposomes;

(d) removing unbound affinity liposomes;

(e) releasing encapsulated enzyme activator from the interior of affinity liposomes bound to (A) and/or (C);

(f) bringing the immobilized, inactive enzyme molecules into contact with enzyme activator released from the interior of the affinity liposomes and restoring the activity of the enzyme molecules;

(g) adding the substrate being convertible into reporter molecules to the enzyme molecules; and

(h) measuring the reporter molecules formed by activated enzyme molecules.

Preferably, the enzyme activator is released from the interior of the liposome by increasing the ambient temperature or by adding a liposome-lysing agent. The method further involves adding polymeric carrier molecules capable of specifically binding to (A) and/or (C) in conditions where (A) and (C) are bound to each other but not to free (C), the polymeric carrier molecules being the components bound or later to be bound to the affinity liposomes by providing more than one binding site for the affinity liposomes per each of the polymeric carrier molecules and removing unbound polymeric carrier molecules, before adding enzyme activator containing affinity liposomes, and where the affinity liposomes added are capable of specifically binding to (A)-bound polymeric carrier molecules. Optionally, detecting an analyte (preferably a low molecular weight (A)) in liquid sample comprises:

(a) providing (I) comprising (C) immobilized on a solid support and capable of specifically binding (A), affinity liposomes containing encapsulated enzyme activator and having (A) to be analyzed attached to their surface, enzyme molecules in an inactive condition immobilized on a solid support, and a substrate being convertible to reporter molecules by the enzyme molecules in activated condition;

(b) mixing the sample with a limited quantity of the affinity liposomes;

(c) contacting the mixture with the immobilized (C);

(d) removing unbound affinity liposomes;

(e) releasing encapsulated enzyme activator from the interior of affinity liposomes bound to (C);

(f) bringing the immobilized, inactive enzyme molecules into contact with enzyme activator released from the interior of the affinity liposomes and restoring the activity of the enzyme molecules;

(g) adding the substrate being convertible into reporter molecules to the enzyme molecules; and

(h) measuring the reporter molecules formed by activated enzyme molecules.

Alternatively, detecting (A) in a liquid sample, involves:

(a) providing (C) immobilized on a solid support and capable of specifically binding (A), (A) molecules labeled with an affinity component capable of binding affinity liposomes containing surface-attached complementary affinity components, affinity liposomes containing encapsulated enzyme activator and having the complementary affinity components attached to their surface, enzyme molecules in an inactive condition immobilized on a solid support, a substrate being convertible to reporter molecules via the enzyme molecules in activated condition;

(b) mixing the sample with a limited quantity of (A) molecules;

(c) contacting the mixture with the immobilized (C);

(d) adding affinity liposomes;

(e) removing unbound affinity liposomes;

(f) releasing encapsulated enzyme activator from the interior of affinity liposomes bound to labeled (A); and

(g) carrying out steps (f)-(h) of the previous method (all claimed).

ADVANTAGE - The enzymatic amplification system allows signal enhancement by several orders of magnitude. Since affinity liposomes allow encapsulation of large amounts of enzyme activators, multiple enzyme molecules are activated upon release of the enzyme activators from each (A)-bound affinity liposome. Only partial purified enzyme preparations are used, and the size of the solid-phase area can be easily adjusted to accommodate additional contaminating, enzymatically inactive proteins. As a result (I) reduces costs and provides a new technique for highly efficient signal amplification.

DESCRIPTION OF DRAWING(S) - The figure shows detection of target nucleic acids using affinity liposomes containing encapsulated Zn2+ ions on surface attached intercalating agents.

Solid support (1)

Surface bound capture oligonucleotide (2)

EQUIVALENT-ABSTRACTS:

POLYMERS

Preferred System: The polymeric carrier molecules are chosen from derivatives of synthetic or natural polymers including polysaccharides, poly(amino acids), poly(vinyl alcohols), poly (vinylpyrrolidinones), poly(acrylic acids), polyurethanes, polyphosphazenes and copolymers of the polymers.

BIOTECHNOLOGY

Preferred System: In (I), the reporter molecules are optical detectable molecules and the reporter system is an optical reporter system. The reporter molecules are electrochemically detectable molecules, where (I) further comprises an electrochemical sensor comprising a closely spaced array of thin film noble metal electrodes for voltammetric detection of the reporter system. The electrochemical sensor is a microelectronic interdigitated array of electrodes, where anodes and cathodes have a width of 100 - 800 nm and the electrodes are spaced apart from each other with a distance of 5 - 5000 nm. The (C) and inactive enzyme molecules are immobilized on different supports, where the (C) are immobilized on a substantially flat support, preferably on a structure of a container in which detection is intended to take place, and the inactive enzyme molecules are immobilized on a support which may be introduced into the container, preferably having the shape of a strip or of beads, or vice versa. The surface-attached affinity component(s) of the affinity liposomes is capable of specifically binding to captured (A) chosen from nucleic acid-reactive components, antigens including haptens, immunoglobulins and its fragments, non-immunoglobulin binding proteins, peptides, non-biologic binding molecules and binding to the

captured (A) in a structure-restricted manner. The surface-attached affinity component(s) of the affinity liposomes is a nucleic acid-reactive component chosen from single-stranded RNA and DNA, single-stranded oligonucleotides, preorganized oligonucleotide structures including peptide nucleic acid (PNA) analogs, intercalating agents, intercalating agents conjugated to single-stranded oligonucleotides or nucleic acids, immunoglobulins or its fragments with specificity for double- and/or triple-stranded nucleic acids, and non-immunoglobulin proteins capable of specifically binding to double-stranded nucleic acids. The surface-attached affinity component(s) of the affinity liposomes is attached to the surface of the affinity liposomes by a spacer molecule chosen from oligoethylene glycol derivatives and monomers to oligomers of aminoalkyl acid derivatives. The inactive enzyme molecules are chosen from apometalloenzymes and non-metalloenzymes both of which are only catalytically active in the presence of certain metals, inactive deletion mutant enzymes which require complementation by certain polypeptides to be catalytically active, and incomplete, inactive enzyme complexes which require the presence of additional enzyme subunits to be catalytically active. The substrate is chosen from phosphomonoester derivatives of optical reporter molecules or redox mediators for metallophosphatases, phosphodiester derivatives of optical reporter molecules or redox mediators of venom exonucleases, amino acid derivatives of optical reporter molecules or redox mediators for metalloaminopeptidases and metallocarboxypeptidases, glycoside derivatives of redox mediators for glycosidases including galactosidase derivatives of redox mediators for beta-galactosidase, galactosidase derivatives of

optical reporter molecules for beta-galactosidase, and NAD+as a cofactor of metallodehdrogenases. The derivative of redox mediators are chosen from p-aminophenol, catechol, dopamine, methoxytyraminin and their derivatives, aromatic compounds with more than one aromatic ring structure including naphthol and anthracene derivatives, and heterocyclic aromatic compounds including serotonin, hydroxyindol acetic acid, or their derivatives. The covalently linked affinity component of the polymeric carrier molecules capable of specifically binding to the affinity liposomes, and the surface-attached affinity components of the affinity liposomes are chosen from haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin and streptavidin. The complexes of (II) as described in (iii) are formed from affinity liposomes containing two types of surface-attached affinity components, type I surface-attached affinity components being capable of specifically binding to (A) and/or the (C) in a condition where (A) and (C) are bound to each other, but not to free (C), and the type II surface-attached affinity components being capable of mediating the complexation of affinity liposomes by bridging molecules which have two binding sites for type II affinity components. Optionally, the type II surface-attached affinity components are capable of mediating the complexation of affinity liposomes by polymeric carrier molecules with immobilized affinity components providing two binding sites for type II affinity components. The surface-attached type II affinity components capable of mediating the complexation of (II) by bridging molecules is chosen from haptens, enzyme inhibitors and biotin. The bridging molecules are chosen from molecules with more than one binding site, preferably chosen from:

(i) bi- or oligovalent anti-hapten antibodies, or its fragments, conjugates or fusion constructs;

(ii) inhibitor-binding enzymes, its conjugates, fusion constructs; and

(iii) avidin or streptavidin.

The surface-attached type II affinity components capable of mediating the complexation of (II) by polymeric carrier molecules, and the affinity components immobilized on the polymeric carrier molecules are chosen from haptens, anti-hapten antibodies, enzyme inhibitors, inhibitor-binding enzymes, biotin, avidin and streptavidin. Optionally, the complexes of (II) in (iii) are formed from two types of affinity liposomes, type I affinity liposomes containing two surface-attached affinity components capable of specifically binding to captured (A)s, and type II affinity liposomes containing two surface-attached affinity components capable of specifically binding to the surface-attached affinity components of type I affinity liposomes. Preferably, the type I affinity liposomes are chosen from oligonucleotides capable of forming specific helical complexes with specific target nucleic acids or their amplicons, and the affinity components of type II affinity liposomes are chosen from oligonucleotides capable of forming specific helical complexes with the oligonucleotides of type I affinity liposomes. Alternatively, the affinity components of type I affinity liposomes are chosen from molecules capable of specifically binding to captured (A)s including immunoglobulins or its fragment, non-immunoglobulin and peptides, and the affinity components of type II affinity liposomes are chosen

from immunoglobulins or fragments with specificity for affinity components of type I affinity liposomes. Complexes of (III) in (iv) are formed from affinity liposomes containing two types of surface-attached affinity components such as surface-attached type I affinity components, and type II affinity components which are capable of mediating the complexation of affinity liposomes by bridging molecules. Optionally, the complexes of (III) are formed from affinity liposomes containing two types of surface-attached affinity components such as surface-attached type I affinity components, and type II affinity components which are capable of mediating the complexation of affinity liposomes by polymeric carrier molecules having immobilized affinity complexes.

Kits for carrying out the assay procedure of detecting (A) in liquid is also disclosed.

Detection of mouse immunoglobulin (Ig)G using enzyme activator-containing affinity liposomes was carried out as follows. Rat anti-mouse IgG was immobilized onto tresyl-activated Sepharose beads as described in Meth.Enzymol.104,56,1984. Zinc-free alkaline phosphatase was prepared as described in Biochemistry 13, 3754, 1974. For immobilization of the inactive enzyme molecules onto the surface of the gold electrodes, the electrodes were derivatized with a heterobifunctional reagent containing a terminal mercapto residue and a carboxy group. The carboxy group was used to couple zinc-free alkaline phosphatase in a carbodiimide-mediated reaction. Biotinylated affinity liposomes were prepared by injection method (Biochim.Biophys. Acta 298, 1015, 1973) from a lipid mixture of dimyristoylphosphatidylcholine, cholesterol, dicetylphosphate at a molar ratio of 5:4:1, and N-

biotinyldipalmitoyl-L-alpha-phosphatidylethanolamine (B-PE) at a concentration of 0.1 mol % of total lipid. To prepare liposomes, 2 micromoliters of stock lipid mixture in chloroform was evaporated under a stream of nitrogen and then placed in a vacuum desiccator overnight. The lipid was resolubilized in 50 microliters of dry isopropanol and injected with a syringe into 1 ml of a 2 M ZnSO4 solution which was mixed by vortex. Liposomes of uniform size were formed spontaneously by this method. Unencapsulated ZnSO4 was removed by gel filtration. p-aminophenylphosphate (PAPP) was synthesized from p-nitrophenylphosphate as described in Biochemistry, 20, 1606, 1981. The analytical system was washed with several volumes of 50 mM N-(2-OH-ethyl-) piperazine-N'-(2-ethanesulfonic acid) (HEPES), 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2, and captured mouse IgG was detected by the addition of 50 microliters rat anti-mouse IgG-streptavidin conjugate in 50 mM HEPES, pH 7. After an incubation for 5 minutes at room temperature, the immunoreaction chamber was washed again with several volumes of 50 mM HEPES, pH 7, followed by the addition of 50 microliters biotinylated affinity liposomes containing ZnSO4. After an incubation of 5 minutes at room temperature, the immunoreaction chamber was washed with several volumes of 20 mM metal-free sodium carbonate buffer, pH 9.5. Finally, the valve connecting the immunoreaction chamber with the flow chamber was opened and bound affinity liposomes were lysed by the addition of 100 microliters of 20 mM metal-free sodium carbonate buffer, pH 9.5, containing 0.01 % Triton X-100 and 5 mM PAPP. Activated alkaline phosphatase was detected by redox recycling of generated p-aminophenol (PAP). The PAP enzymatically generated from PAPP was oxidized to

quinoneimine at the anode (+250 mV) yielding an oxidation current. At the cathode (-50 mV) the quinoneimine was reduced to PAP. Using redox cycling for the detection of PAP, the detection limit was in the range of 50 nM corresponding to 2.5 pmol per 50 microliters. Considering the high turn over number of alkaline phosphatase (approximately 3000 s-1), an incubation period of 120 s allowed the detection of alkaline phosphatase in the range of 10 attomol (10 to the power of -17 mol). Since the release of approximately 10 to the power of 5 Zn2+ ions from a single bound affinity liposome reactivates many immobilized alkaline phosphatase molecules. The described assay configuration allowed the detection of mouse IgG in the zeptomol range (less than 100 fg/50 microliters).

CHOSEN-DRAWING: Dwg.1A/2

TITLE-TERMS: REPORT SYSTEM DETECT ANALYTE LIQUID COMPRISE SOLID SUPPORT CONTAIN IMMOBILISE CAPTURE MOLECULAR INACTIVE ENZYME ACTIVATE AFFINITY SUBSTRATE

DERWENT-CLASS: A89 B04 D16 S03

CPI-CODES: A12-V03C2; A12-W11L; B04-B04C; B04-C02; B04-C03B; B04-C03D; B04-E01; B04-E05; B04-G01; B04-N04; B11-C08B; B12-K04E; D05-H09; D05-H10;

EPI-CODES: S03-E14H;

CHEMICAL-CODES: Chemical Indexing M1 *01*
Fragmentation Code H4 H401 H481 H7 H713 H721 H8 M210 M212 M272 M281 M320 M423 M424 M431 M510 M520 M530 M540 M740 M782 N102 Q120 Q233 Specific Compounds RA01EA Registry Numbers 104492

Chemical Indexing M1 *02*
Fragmentation Code F012 F013 F423 H7 H715 H721 J5 J521 L9 L941 M210 M212 M240 M281 M320 M423 M424 M431 M510 M521 M530 M540 M740 M782 N102 Q120 Q233 Specific Compounds RA00D5 Registry Numbers 1062

Chemical Indexing M1 *03*
Fragmentation Code H7 H714 H721 J0 J011 J1 J171 M210 M212 M262 M281 M320 M423 M424 M431 M510 M520 M530 M540 M630 M740 M782 N102 Q120 Q233 Specific Compounds RA02L0 RA037T Registry Numbers 104380 199392

Chemical Indexing M1 *04*
Fragmentation Code K0 L4 L463 L499 M280 M312 M313 M314 M315 M323 M332 M342 M383 M393 M423 M424 M431 M510 M520 M530 M540 M620 M740 M782 N102 Q120 Q233 Specific Compounds R16492 Registry Numbers 104486

Chemical Indexing M1 *05*
Fragmentation Code M423 M424 M431 M740 M750 M782 N102 P831 Q120 Q233 Specific Compounds RA012P Registry Numbers 105730

Chemical Indexing M1 *06*
Fragmentation Code M423 M424 M431 M740 M750 M782 N102 P831 Q120 Q233 Specific Compounds RA00NS Registry Numbers 93605

Chemical Indexing M1 *07*
Fragmentation Code M417 M423 M424 M431 M740 M750 M782 N102 P831 Q120 Q233 Specific Compounds RA013I Registry Numbers 184610

Chemical Indexing M1 *08*
Fragmentation Code M417 M423 M424 M431 M740 M750 M782 N102 P831 Q120 Q233 Specific Compounds RA00GC Registry Numbers 184598

Chemical Indexing M1 *09*
Fragmentation Code M417 M423 M424 M431 M740 M750 M782 N102 P831 Q120 Q233 Specific Compounds RA00C8 Registry Numbers 184587

Chemical Indexing M1 *10*
Fragmentation Code M417 M423 M424 M431 M740 M750 M782 N102 P831 Q120 Q233 Specific Compounds RA00H1 Registry Numbers 184611

Chemical Indexing M1 *11*
Fragmentation Code M417 M423 M424 M431 M740 M750 M782 N102 P831 Q120 Q233 Specific Compounds RA00H3 Registry Numbers 184616

Chemical Indexing M1 *12*
Fragmentation Code M417 M423 M424

M431 M740 M750 M782 N102 P831 Q120 Q233 Specific Compounds RA0121 Registry Numbers 184614

Chemical Indexing M1 *13* Fragmentation Code D011 D012 E720 J0 J011 J1 J171 J5 J521 L9 L921 M280 M314 M321 M332 M342 M372 M391 M412 M423 M424 M431 M511 M520 M530 M540 M740 M750 M782 N102 P831 Q120 Q233 Ring Index Numbers 00945 Specific Compounds R00172 Registry Numbers 2408

Chemical Indexing M1 *14* Fragmentation Code M423 M424 M431 M740 M750 M782 N102 P831 Q120 Q233 Specific Compounds RA0497 Registry Numbers 88056

Chemical Indexing M6 *15* Fragmentation Code P831 Q120 Q233 R515 R521 R528 R621 R622 R624 R627 R630 R631 R632 R639

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Polymer Index [1.2]
018 ; G3623*R P0599 D01;

Polymer Index [1.3]
018 ; G2062*R D01 D60 F07 F35; P0635*R F70 D01; H0011*R; H0011*R;

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Polymer Index [1.5]
018 ; G0635 G0022 D01 D12 D10 D23 D22 D31 D41 D51 D53 D58 D75 D86 F71; H0000; H0011*R;

Polymer Index [1.6]
018 ; P1592*R F77 D01;

Polymer Index [1.7]
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